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# Mixture toxicity - single-substance testing to ecosystem effect assessment

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*“To accomplish great things we must not only act, but also dream; not only plan, but also believe.”*

— Anatole France, Works of Anatole France

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# Glossary

| | |
|---|---|
| ANOVA | Analysis of Variance |
| CA | Concentration Addition |
| CAS | Chemical Abstracts Service |
| ECx | Effect Concentration (x % effect within test population) |
| EU | European Union |
| IA | Independent Action |
| LCx | Lethal Concentration (x % mortality within test population) |
| MoA | Mode of Action |
| NOEC | No observed effect concentration |
| OECD | Organization for Economic Co-operation and Development |
| REACH | EU regulation for Registration, Evaluation, Authorization and Restriction of Chemical substances |
| SE | Standard error |
| TU | Toxic Unit |
| US EPA | United States Environmental Protection Agency |

# Preface

This work is intended to contribute to the effect assessment of pesticide mixtures in ecotoxicological testing by comparing short-term single-species testing to long-term multiple-species-mesocosm testing.

The first chapter contains an introduction providing essential background information on mixture toxicity and the approaches that are commonly used in pesticide regulation and research, as well as an overview of the insecticides that were used for this project, the importance of invertebrates in toxicity testing, and an introduction to mesocosm test systems. Following the introduction, specific research topics on single-species testing using single chemicals as well as mixtures using two standard toxicity test species, *C. dilutus* and *H. azteca*, as well as mixture effects on an exemplary aquatic invertebrate community are presented (chapters 2 – 4). Each specific chapter was published (chapter 2 and 3) or submitted (chapter 4) as an autonomous research paper in a slightly modified form (according to the different journal requirements). In a fifth chapter a general discussion on the three research topics ties the three research topics together by focusing on the advantages and disadvantages of the approaches, the different endpoints investigated, and how gained information can be applied in future mixture toxicity investigations. In a final conclusion (chapter 6), the research findings are viewed in the context of a more complex scale involving fish and an entire ecosystem.

## Summary

Contaminants are one of the major threats to biological diversity worldwide, with aquatic ecosystems being polluted by chemicals originating from various sources. Pesticide mixtures affect aquatic communities, however most data used in risk assessment of pesticides are based on single species tests using single substances, at concentrations that are usually not generally environmentally realistic. Given the increased use of insecticides and their detrimental effects on non-target organisms of aquatic ecosystems and food webs, understanding complex mixtures of contaminants is one of the major challenges that the field of ecotoxicology is currently facing. To improve the understanding of pesticide mixture effects, this project investigated the advantages and disadvantages of single-species laboratory toxicity tests as well as the use of outdoor, multiple-species mesocosms for assessing contaminant mixture effects.

First, single species tests were conducted using single chemicals to compare the effectiveness of *C. dilutus* and *H. azteca* toxicity tests to detect toxicity caused by four current-use insecticides: three pyrethroids, bifenthrin, permethrin, and cyfluthrin, and one organophosphate, chlorpyrifos. The three pyrethroids were more toxic than the organophosphate chlorpyrifos for both species. Bifenthrin was most toxic to *H. azteca* survival and growth and cyfluthrin was most toxic to *C. dilutus*, however, cyfluthrin had the greatest effect on motility on both *H. azteca* and *C. dilutus*. The evaluated concentrations of chlorpyrifos did not affect *C. dilutus* motility nor growth, but significantly impacted *H. azteca* growth. Motility was the most sensitive endpoint in assessing sublethal effects at low concentrations for both species, while growth was a good indicator of toxicity for all four pesticides for *H. azteca*.

Secondly, lambda-cyhalothrin, permethrin, and chlorpyrifos were applied individually and in tertiary mixtures, and assessed in 10-day toxicity tests using *C. dilutus*, to investigate the effects of the three pesticides on sublethal endpoints such as growth and motility by utilizing a toxic units (TU) approach; based on median lethal concentrations (LC50) for each compound. The concepts of independent action and concentration addition were used to compare predicted mixture toxicity to

observed mixture toxicity. Increased immobility resulted from mixture concentrations ≥ 1 TU (7.45 ng/L lambda-cyhalothrin x 24.90 ng/L permethrin x 129.70 ng/L chlorpyrifos), and single pesticides concentrations ≥ 0.25 TU (5.50 ng/L lambda-cyhalothrin, 24.23 ng/L permethrin, 90.92 ng/L chlorpyrifos, respectively). Growth was inhibited by pesticide mixtures ≥ 0.125 TU (1.04 ng/L lambda-cyhalothrin x 3.15 n/L permethrin x 15.47 ng/L chlorpyrifos), and singly by lambda-cyhalothrin ≥ 0.25 TU (5.50 ng/L), and permethrin ≥ 0.167 TU (18.21 ng/L). The no observed effect concentrations (NOEC) for immobility and growth, for both mixture and single-pyrethroid exposure, were up to 8.0 and 12.0 times respectively lower than the corresponding NOEC for survival. The median effective concentrations (EC50) for growth (mixture and single-pyrethroid exposure) were up to 7.0 times lower than the respective LC50.

Lastly, the long-term contaminant mixture effects on invertebrate community structure, function, and biomass, encompassing different life stages and their seasonal development were investigated. By conducting a 6-month, multispecies outdoor mesocosm study, with multiple applications of environmentally relevant and laboratory-defined effective concentration treatments, the long-term effects of exposure to the insecticides lambda-cyhalothrin, permethrin, and chlorpyrifos was assessed. In addition, the fate of the contaminants, in both the water column and sediment, were monitored to assess how this passage affects the species living in the different habitats within the mesocosms. The most sensitive taxa were the snail *Radix*, the amphipod *H. azteca*, the water flea *Daphnia magna*, and copepods. Environmentally relevant concentrations had greatest effects on zooplankton communities, and caused acute effects on *D. magna* and *H. azteca* (occurring 24h after application) while lag-times were more pronounced in *Radix* snails and copepods indicating chronic sublethal responses. Pyrethroids rapidly dissipated from the water column, while chlorpyrifos was detectable, even after six weeks of exposure. Twelve of fifteen macroinvertebrate and ten of sixteen zooplankton taxa responded significantly to environmentally relevant contaminant exposures.

By using laboratory-based toxicity tests it was possible to determine ecologically relevant sublethal effects such as growth and motility under controlled conditions within a very short period of time. Mesocosms on the other hand allowed to evaluate long-term community and food-web effects,

and as such represented a much more realistic exposure scenario. Both approaches provided essential information for understanding mixture toxicity and evaluating their effects on aquatic ecosystems, which can be applied in risk-assessments of contaminants of concern. Especially the integration of sublethal endpoints in ambient water monitoring and pesticide regulation efforts could improve identification of low-level pesticide concentrations that may eventually cause negative effects on food webs and community structure in aquatic environments.

## Zusammenfassung

Schadstoffe stellen eine der größten Gefährdungen für die weltweite Biodiversität dar, wobei besonders aquatische Ökosysteme als Senken fungieren und Chemikalien verschiedensten Ursprungs ausgesetzt sind. Es ist bekannt, dass Pestizidmischungen aquatische Lebensgemeinschaften negativ beeinflussen können, dennoch beruhen Daten für die Risikobewertung von Pestiziden meist nur auf Einzelartentoxizitätstests, die oft nur mit einer einzelnen Substanz und in Konzentrationen, die nicht umweltrelevant sind, durchgeführt werden. Aufgrund zunehmender Verwendung von Insektiziden und den damit verbundenen schädlichen Auswirkungen auf Nichtzielarten in aquatischen Ökosystemen und Nahrungsnetzen, stellt das Verständnis von komplexen Pestizidmischungen eine der aktuell größten Herausforderungen in der Ökotoxikologie dar. Um das Verständnis der Auswirkungen von Pestizidmischungen zu verbessern, wurden in dieser Arbeit die Vor- und Nachteile von Einzelartentoxizitätstests im Labor im Vergleich zu Multi-Spezies-Mesokosmentests im Freiland untersucht.

In einem ersten Schritt wurden Einzelartentests mit Einzelsubstanzen im Labor durchgeführt, um die Eignung von *Chironomus dilutus* und *Hyalella azteca* für die Toxizitätsbewertung von vier Insektiziden (den Pyrethroiden Bifenthrin, Permethrin, und Cyfluthrin, und dem Organophosphat Chlorpyrifos) zu vergleichen. Für beide Testarten waren die drei Pyrethroide toxischer als das Organophoshphat Chlorpyrifos. Dabei war Bifenthrin am schädlichsten für die Überlebensrate und das Wachstum von *H. azteca* und Cyfluthrin am schädlichsten für *C. dilutus*, wobei Cyfluthrin den größten Effekt auf die Motilität sowohl von *H. azteca* als auch von *C. dilutus* hatte. Die untersuchten Konzentrationen von Chlorpyrifos hatten keinen Einfluss auf die Motilität oder das Wachstum von *C. dilutus*, beeinträchtigten aber das Wachstum von *H. azteca*. Motilität war der sensitivste Endpunkt für die Bestimmung von sublethalen Effekten bei niedrigen Konzentrationen für beide Arten, während Wachstum ein guter Indikator für die Toxizität von allen vier Pestiziden auf *H. azteca* war.

In einem zweiten Schritt wurden die individuellen Effekte von Lambda-cyhalothrin, Permethrin und Chlorpyrifos im Vergleich zu Dreifachmischungen untersucht. Dabei wurden 10-Tages-

Toxizitätstests mit *C. dilutus* unter Anwendung einer Toxic Unit (TU) Methode, welche auf der mittleren letalen Konzentration (LC50) für jede Substanz basiert, durchgeführt und die subletalen Endpunkte Wachstum und Motilität analysiert. Die Konzepte der Unabhängigen Wirkung und Konzentrationsaddivität wurden verwendet, um die berechnete Mischungstoxizität mit der beobachteten Toxizität zu vergleichen. Erhöhte Immobilität wurde sowohl für Mischungskonzentrationen ≥ 1 TU (7.45 ng/L Lambda-cyhalothrin x 24.90 ng/L Permethrin x 129.70 ng/L Chlorpyrifos), als auch für Einzelkonzentrationen ≥ 0.25 TU (5.50 ng/L Lambda-cyhalothrin, 24.23 ng/L Permethrin, bzw. 90.92 ng/L Chlorpyrifos) beobachtet. Das Wachstum von *C. dilutus* war bei Mischungskonzentrationen ≥ 0.125 TU (1.04 ng/L Lambda-cyhalothrin x 3.15 n/L Permethrin x 15.47 ng/L Chlorpyrifos) und Einzelkonzentrationen von Lambda-cyhalothrin ≥ 0.25 TU (5.50 ng/L) und Permethrin ≥ 0.167 TU (18.21 ng/L) gehemmt. Sowohl für die Dreifachmischung als auch für die einzelnen Pyrethroide waren die höchsten getesteten Konzentrationen ohne beobachtete schädliche Wirkung (No observed effect concentration, NOEC) auf Immobilität und Wachstum bis zu 8.0 bzw. 12.0 mal niedriger als die entsprechenden NOEC-Konzentrationen für die Überlebensrate. Die mittleren Effektkonzentrationen (EC50) für Wachstum (Mischung und einzelne Pyrethroide) waren bis zu 7.0 mal niedriger als die entsprechenden LC50 Werte.

Um die Langzeiteffekte von Pestizidmischungen auf die Struktur, Funktion und Biomasse von Invertebratengemeinschaften, unter Berücksichtigung der verschiedenen Lebensstadien und der saisonalen Entwicklung, zu untersuchen, wurde eine sechsmonatige Mehrarten-Mesokosmenstudie im Freiland durchgeführt. Die verschiedenen Belastungsstufen der Mischungen der Insektizide Lambda-cyhalothrin, Permetrhin und Chlorpyrifos basierten auf umweltrelevanten Konzentrationen und auch auf Konzentrationen, die in vorausgehenden Laborversuchen auf Einzelarten Wirkungen zeigten. Zusätzlich wurde die Dauer des Verbleibs der Insektizide sowohl in der Wassersäule als auch im Sediment gemessen, um den Einfluss dieser Passage auf die Arten, die in den verschiedenen Habitaten der Mesokosmen leben, differenzieren zu können. Für zwölf von fünfzehn Makroinvertebraten und zehn von sechzehn identifizierten Zooplanktontaxa wurden signifikante Effekte durch die Pestizidbelastung gemessen. Die sensitivsten Taxa waren die Schneckenart *Radix*,

der Amphipod *H. azteca*, der Wasserfloh *Daphnia magna* und die Gruppe der Copepoden. Umweltrelevante Konzentrationen hatten den stärksten Einfluss auf die Zooplanktongemeinschaft und verursachten innerhalb von 24h nach der Applikation akute Effekte auf *D. magna* und *H. azteca*, während verzögerte Effekte für die *Radix* Schnecken und die Copepoden beobachtet wurden. Diese verzögerten Effekte weisen auf potentielle chronische subletale Effekte hin. Die Pyrethroide waren nach kurzer Zeit nicht mehr in der Wassersäule messbar, während Chlorpyrifos bis zu 6 Wochen nachweisbar war.

Durch die Anwendung von laborbasierten Toxizitätstests war es möglich, ökologisch relevante subletale Effekte auf Wachstum und Motilität unter kontrollierten Bedingungen in 10-Tagestests zu bestimmen. Andererseits ermöglichte die Einbeziehung von Mesokosmenversuchen im Freiland die Abschätzung von Langzeiteffekten auf ganze Biozönosen und das Nahrungsnetz, weshalb dieser Versuchsansatz eine realistischere Einschätzung der Effekte darstellte als die Einzelartentests. Beide Versuchsansätze lieferten essentielle und komplementäre Informationen, um die Mischungstoxizität besser verstehen und die daraus resultierenden Effekte auf aquatische Ökosysteme bewerten zu können, welche in der Risikobewertung von Schadstoffen Anwendung finden sollten. Besonders die Integration von subletalen Endpunkten in die Gewässerüberwachung und Pestizidregulierung kann die wirkungsbezogene Identifizierung von niedrigen Pestizidkonzentrationen, welche langfristig negative Effekte auf die Nahrungsnetze und Gesellschaftsstrukturen in Gewässern verursachen können, erleichtern.

# 1. Introduction

Contaminants pose a major threat to freshwater biodiversity (Dudgeon *et al.* 2006; Geist 2011; Connon *et al.* 2012), as aquatic ecosystems worldwide are polluted by chemicals originating from various sources (Scholz *et al.* 2012). Contaminants can enter surface waters via direct overspray or spray drift (Solomon and Thompson 2003), wastewater treatment plant effluent discharge (Parry and Young 2013), runoff from urban gardens, agriculture (Werner *et al.* 2004), and hard surfaces such as pavements (Huang *et al.* 2005; Vijver *et al.* 2008). Natural systems are thus exposed to a number of contaminants that alter species abundance and diversity (Fleeger *et al.* 2003). While much research is conducted on the effects of individual contaminants, understanding the effects posed by contaminant mixtures remains one of the most pressing needs in the field of environmental risk assessment.

## 1.1 Environmental Risk Assessment

Environmental risk assessment (ERA) is defined as the procedure by which the adverse effects of pollutants and other anthropogenic activities on ecosystems and their components are estimated with a known degree of certainty using scientific methodologies (Depledge and Fossi 1994).

The European regulatory framework for chemicals (REACH) stipulates that standardized ecotoxicological hazard assessments should be conducted using organisms from different trophic levels (primary producers, primary and secondary consumers) (Beyer *et al.* 2014). According to the Organization for Economic Co-operation and Development (OECD 1989) these assessments can be divided into three stages. *Preliminary effects assessment* is the first stage (Tier-1) at which only short-term toxicity data are available such as quantitative structure-activity relationship (QSAR) estimates or median lethal/effect concentration (LC50 or EC50) values derived from laboratory exposures. In QSAR modeling, chemical effects are predicted based on supposed relationships between physico-chemical properties or theoretical molecular descriptors of chemicals (Escher and Hermens 2002). A median lethal concentration (LC50) is the estimated concentration of the test material that will kill or immobilize 50% of the test organisms in a predetermined period of time. Similarly, median effect concentrations (EC50) can be calculated for any specified effect. In tier-2, a *refined or intermediate*

*effects assessment* can take place if a few no observed effect concentrations (NOECs) from chronic tests are available. A NOEC is the highest test concentration below which no adverse effect occurs (Van Der Hoeven 1997). Finally, the *comprehensive effects assessment* is the third stage (tier-3) at which field studies, multispecies toxicity studies (or many chronic test results) are taken into account (OECD 1989).

The large number of existing chemicals does not allow an in-depth-risk assessment at the level of disturbance of an ecosystem. Thus, the generic risk assessment scheme has developed into a system, in which the information gained from each tier for three trophic levels (algae, daphnids, fish), in conjunction with an assessment factor (in the range of 1-1000), can be used to calculate the predicted no effect concentration (PNEC). The PNEC represents an estimate of the putative effects that each contaminant may have in specific ecosystem situations (Van Leeuwen *et al.* 1996; Backhaus and Faust 2012). The calculation of the predicted environmental concentration (PEC) gives an estimate of the level of exposure for a given scenario and is thus essential for an initial indication of negative impact (Kelly *et al.* 2003). The quotient of the PEC of a chemical and its toxic potential given by the PNEC value results in a PEC/PNEC ratio (the Risk Quotient, RQ) which is widely used as a standardized measure of risk in environmental risk assessment (van der Oost *et al.* 2003).

Other approaches based on species sensitivity distributions (SSDs) or detailed toxicokinetic and/or –dynamic modeling can be used in situations where a considerable amount of ecotoxicological information is available for every single component in the mixture (Ashauer *et al.* 2011; Rubach *et al.* 2012). SSDs quantify the fraction of species that are potentially affected in contaminated environmental habitats using sensitivity data of several test species (Aldenberg and Jaworska 2000; Forbes and Calow 2002; Wheeler *et al.* 2002). However, REACH requests only three ecotoxicological data sets for most compounds, which are considered to be insufficient for the estimation of SSDs or more elaborate modeling approaches (Backhaus and Faust 2012).

### 1.2 Mixture Toxicity – Implications and Approaches

The aspects of combined effects have not yet been implemented in environmental risk assessment in a standardized manner, nor has the combined effect issue become an integral part of chemical

regulation. Since organisms in polluted environments are typically exposed to a complex mixture of chemical contaminants, exposure may sometimes elicit toxic effects even though the individual stressors are present at very low concentrations, or below NOEC (Silva *et al.* 2002; Kortenkamp 2008), and for many chemicals, at concentrations below the limit of analytical detection. As such, risk assessment on single compounds may potentially underestimate adverse effects of environmental chemical mixtures. Additionally, a combined exposure to chemical and non-chemical factors such as predation and competition can magnify the complexity of multiple stressor situations (Heugens *et al.* 2001; Harwood *et al.* 2009; Geist 2011). Therefore, a simple and robust approach is needed to efficaciously assess the ecotoxicity of chemical mixtures in environmental risk assessment and regulatory toxicology.

Several top-down and bottom-up oriented test strategies are applied to assess mixture effects. Top-down strategies use biological responses to identify causal agents of toxicity in chemical mixtures (Beyer *et al.* 2014). The most relevant are Effect-Directed Analysis (EDA) and Toxicity Identification and Evaluation (TIE) (Besser *et al.* 1998; Burgess *et al.* 2013). EDA uses primarily mechanism-specific *in vitro* bioassay endpoints whereas TIE typically determines active toxicants to whole-organism endpoints (Beyer *et al.* 2014). The TIE procedures were developed by US EPA, and are mainly used for identification and evaluation of contaminants in aqueous samples (de Vlaming *et al.* 2000; Brack *et al.* 2008). In EDA, the fractionation and chemical analyses performed to identify the causes of toxicity may often include contaminant bioavailability; whereas in TIE, toxicant bioavailability is maintained and is considered crucial for identifying the chemicals responsible for toxicity (Beyer *et al.* 2014).

However, both EDA and TIE approaches have limitations when assessing mixture toxicity. Because joint effects of substances can influence each other's toxicity, they result in an almost unlimited number of possible additive (i.e., sum of the toxicities for each mixture compound), synergistic (i.e., greater effect than expected on the basis of additivity predictions) or antagonistic (i.e., lesser effect than expected on the basis of additivity predictions) combinations (Jonker *et al.* 2005; Beyer *et al.* 2014; Cedergreen 2014). By applying bottom-up evaluations, such as concentration

addition and independent action models, the nature and magnitude of combined toxicities can be assessed.

### 1.2.1 Concentration Addition and Independent Action models

Two different models have been defined to make predictions of the combined effects of chemicals, namely concentration addition (CA, also called dose addition or Loewe additivity) and independent action (IA, also called response additivity or Bliss independence) (Greco *et al.* 1995; Altenburger *et al.* 1996; Sühnel 1998). CA occurs when two or more chemicals with similar mode of action (MoA) affect the same target of toxic action (endpoint), whereas IA occurs when two or more chemicals affect the same endpoint but through dissimilar MoAs.

The concept of CA was introduced by Loewe and Muischnek (1926) and Loewe (1927) and can be mathematically explained by:

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i}\right)^{-1} \quad (1)$$

Where $ECx_{mix}$ is the predicted total concentration of the mixture that induces *x*% effect, $p_i$ is the relative fraction of compound *i* present in the mixture, and $ECx_i$ is the concentration of substance *i* provoking a certain effect *x* when applied alone.

The concept of IA was first applied to biological data by Bliss (1939) and can be mathematically explained by:

$$E(c_{mix}) = 1 - \prod_{i=1}^{n}[1 - F_i(c_i)] \quad (2)$$

Where $E(c_{mix})$ denotes the predicted effect (scaled from 0-1) of an *n*-compound mixture, $c_i$ is the concentration of the *i*th compound, and $F_i$ is the effect of that concentration if the compound is applied singly.

Both concepts have been successfully applied in a number of mixture toxicity studies (e.g., Könemann 1981; Hermens *et al.* 1984; Altenburger *et al.* 2000; Faust *et al.* 2001). The majority of these studies have found that mixture toxicity can more reliably be predicted by CA than by IA, while the CA usually generates more conservative predictions (e.g., Backhaus *et al.* 2000; Faust *et al.* 2001; Junghans *et al.* 2003).

However, mixtures containing compounds with either strictly similar, or dissimilar action, are not environmentally realistic, and can thus be considered as extreme special cases (Hewlett and Plackett 1959). Hence, it is not surprising that several studies reported that predictions by CA and IA models are not as clear as expected. In these studies, CA and IA models similarly predicted the effects of mixtures containing chemicals with the same or similar MoAs (Backhaus *et al.* 2004; Syberg *et al.* 2008; Villa *et al.* 2014) and the mixtures containing dissimilarly acting chemicals (Barata *et al.* 2006; Cedergreen *et al.* 2008).

These relationships are caused by, and depend on, different parameters that include: the number of mixture components, their concentration ratios, the response level under consideration, and the slopes of the concentration response curve of the individual toxicants (Drescher and Boedeker 1995).

### 1.2.2 Synergistic and Antagonistic Effects

Common for the CA and IA concepts is the assumption that the chemicals do not interact chemically or affect the toxicity of each other (Loewe and Muischnek 1926; Bliss 1939). If the chemicals do interact, the joint effects might cause deviations of experimental data from the CA and IA model estimates that are commonly identified as synergistic (greater) or antagonistic (lesser) effects compared to the predictions made by either model (see review in Altenburger *et al.* 2003; Belden *et al.* 2007).

Interactions between chemicals can affect a number of processes that are important for the resultant toxicity of a chemical towards an organism: bioavailability, uptake, internal transportation, metabolization, binding at the target site, and excretion (Cedergreen 2014). For example, in a study that investigated the interactions between the herbicide atrazine and the organophosphate insecticide chlorpyrifos, researchers found that the addition of atrazine increased chlorpyrifos uptake by 40% (Belden and Lydy 2000). Mixtures of organophosphates and carbamates have been shown to act synergistically, even though they have the same MoA and therefore supposedly following CA (Laetz *et al.* 2008). An exposure of the fathead minnow *Pimephales promelas* and the amphipod *Hyalella azteca* to binary mixtures of cyfluthrin and imidacloprid also indicated synergistic responses (Lanteigne *et al.* 2015), while another study on imidacloprid using the earthworm *Eisenia fetida* found

anotagonistic effects in tertiary mixtures containing lambda-cyhalothrin, cadmium, and imidacloprid (Wang *et al.* 2015). Antagonistic effects have also been found in a study using *Gammarus fossarum* where sulfur neither affected survival nor the feeding activity of the gammarids but reduced copper-sulfate's toxicity when applied in a binary mixture (Zubrod *et al.* 2014). Similar findings were reported in an exposure of the luminescent bacterium *Vibrio fischeri* to three substituted urea herbicides; monolinuron, linuron, and diuron, which resulted in synergistic, additive and antagonistic effects that varied according to the concentrations of target compounds (Gatidou *et al.* 2015).

These results suggest that the models of CA and IA do not always accurately and reliably predict mixture toxicity and thus more research on mixture toxicity is essential to understand the effects on aquatic ecosystems.

## 1.3 Insecticides of Interest in this Research: Pyrethroids and Organophosphates

### 1.3.1 Mode of Action

Insecticides are substances used to control insect pests in agriculture, medicine, industry, and urban development. The major classes of insecticides include organochlorines, organophosphates (OPs), pyrethroids, neonicotinoids, and ryanoids.

Pyrethroids and OPs were the main focus of this research since both pesticide classes are globally widely applied and estimated to be among the most critical contaminants to aquatic ecosystems. Despite their different modes of action, both classes are neurotoxic and result in hypersensitivity of the nervous system. As neurotoxic compounds they can cause chronic delayed onset toxicity to nerve cells in low doses (Zhang *et al.* 2015).

Pyrethroids are synthetic analogues of the naturally occurring insecticidal esters of chrystanthemic acid (pyrethrins I) and pyrethroic acid (pyrethrins II), originally found in the flowers of *Chrystanthemum cinerafolis*. Pyrethroids impact nerve cell function by interacting primarily with voltage-dependent sodium channels, stimulating nerve cells resulting in repetitive firing of neurons (Shanes 1951; Wang *et al.* 1972). The sodium channels remain open as the pyrethroids impede channel closing either by inactivation or deactivation, and the sodium channels retain the ability to

transport $Na^+$ (Davies *et al.* 2007). Due to their lipophilic nature, biological membranes and tissues readily take up pyrethroids. Exposed organisms may exhibit symptoms of hyperexcitation, tremors, convulsions, followed by lethargy and paralysis (Oros and Werner 2005). Interference of type I pyrethroids (bifenthrin and permethrin) with sodium channel function in the central nervous system, results in restlessness, un-coordination and hyperactivity followed by prostration and paralysis, defined as the T-syndrome (Verschoyle and Aldridge 1980). Type-II compounds (cyfluthrin and lambda-cyhalothrin) have a cyano group at the α-benzylic position (α-carbon of the 3-phenoxybenzyl alcohol) and cause a pronounced convulsive phase resulting in an irreversible depolarization of the nerve axons and terminals, defined as the CS-Syndrome (Verschoyle and Aldridge 1980; Clark and Matsumura 1982; Bloomquist 1996). In addition, recent studies show that some pyrethroids can also have endocrine activity, with metabolites displaying greater endocrine activity than parent compounds (Chen *et al.* 2002; Sun *et al.* 2007; Brander *et al.* 2012). It is important to note that pyrethroids occur mostly as mixtures of stereoisomeric forms, with varying toxicity of the individual isomers (Liu *et al.* 2005).

The main mechanism of action of OPs is the irreversible inhibition of the enzyme acetylcholinesterase (AChE). Although OPs are a broad class of chemicals structurally, they all are acutely toxic to animals through interference with cholinergic nerve transmission. Some OPs can directly cause anticholinesterase effects (phosphate class) or are only toxic after metabolism by cytochrome P-450-dependent monooxygenases; involved in phase I detoxification. This results in bioactivation via the replacement of sulfur with oxygen within the chemical structure of the OP (desulfoxidation), creating a metabolite that is a much stronger inhibitor of AChE (Belden and Lydy 2000). Inhibition of AChE by OPs leads to an accumulation of acetylcholine and subsequent impairment of numerous body functions (Bartling *et al.* 2007).

Due to their broad application as pest control agents, both classes of insecticides have the potential to impact non-target organisms in aquatic environments.

### 1.3.2 Use and Physico-Chemical Properties

Pyrethroids and OPs are widely used for the control of pests in agricultural as well as urban areas (DeLorenzo *et al.* 2014; Hall and Anderson 2014). Both classes are highly nonpolar chemicals of low water solubility, low volatility, and high octanol-water partition coefficients and have high affinity to soil or sediment particles (Table 1).

Table 1 Physical and chemical parameters of pesticides used in this study.

| | Bifenthrin | Permethrin | Cyfluthrin | L-cyhalothrin | Chlorpyrifos |
|---|---|---|---|---|---|
| Molecular Weight (g/mol) | 422.9 | 391.28 | 434.29 | 449.85 | 350.59 |
| Water Solubility (mg/L) | 0.1 | 0.006 | 0.002 | 0.003 | 1.4 |
| Log Koc[1] | 5.4 | 5.4 | 5.1 | 5.5 | 3.93 |
| Vapor Pressure (mm Hg at 25°C) | $1.8x10^{-4}$ | $2.2x10^{-8}$ | $2.03x10^{-9}$ | $1.5x10^{-9}$ | $1.87\ x10^{-5}$ |
| Soil Aerobic Half-life (days) | 96.3 | 39.5 | 11.5 | 42.6 | 109 |
| Soil Anaerobic Half-life (days) | 425 | 197 | 33.6 | | 103 |
| Hydrolysis Half-life (days) | < 30 | >30-242 | 1.84-183 | 8.66->30 | 30 |

Koc = water/organic carbon (Koc) partition coefficient.

Thus, they have little mobility in soils and tend to be associated mainly with sediments of natural water systems (Freed *et al.* 1979; Laskowski 2002).

This research focused on assessing the effects of four pyrethroids (bifenthrin, permethrin, cyfluthrin, and lambda-cyhalothrin) and one organophosphate pesticide chlorpyrifos (Figure 1). These pesticides were selected as exemplary compounds based on the worldwide prevalence of pyrethroids and organophosphates in the environment and their relative toxicities to non-target species (Hintzen *et al.* 2009; Bereswill *et al.* 2013; Li *et al.* 2013).

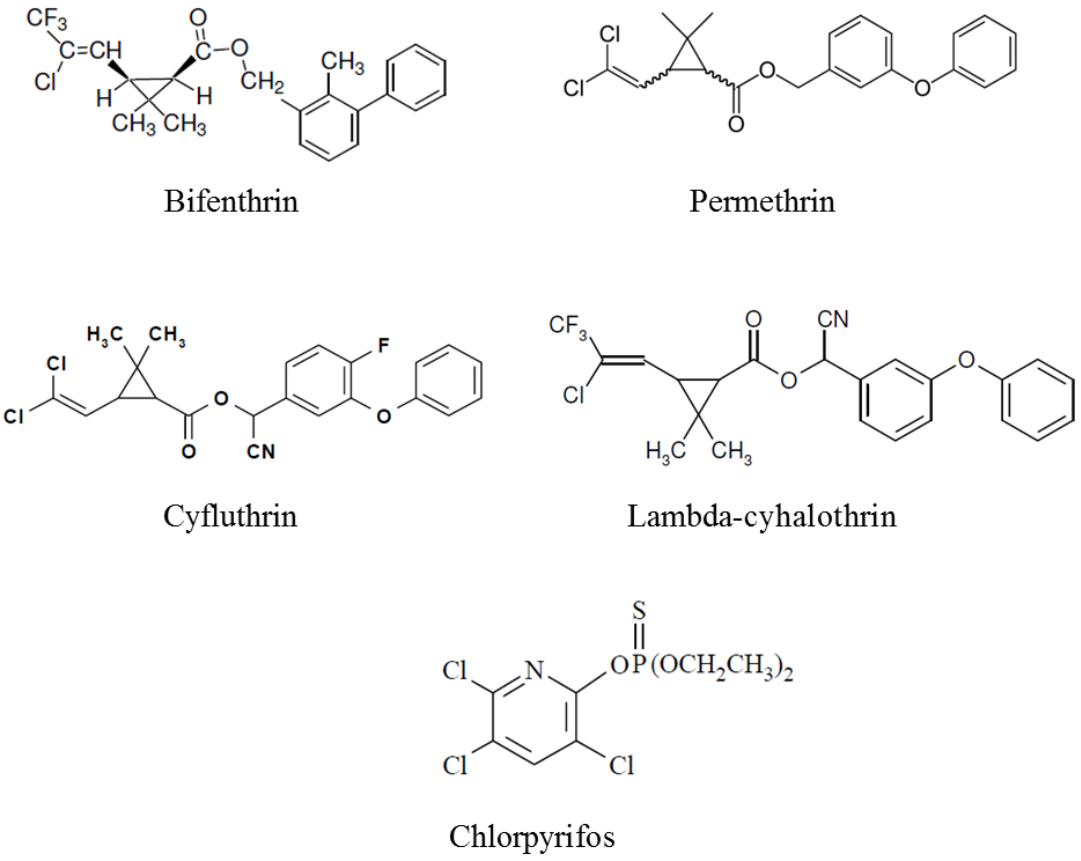


Figure 1 Structural formulas of the pesticides used in this research: bifenthrin, permethrin, cyfluthrin, lambda-cyhalothrin, and chlorpyrifos

Bifenthrin ((2-methyl[1,1'-biphenyl]-3-yl)methyl 3-[(1Z)-2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethylcyclopropanecarboxylate) is a fourth-generation synthetic type-I pyrethroid, characterized by great photostability and insecticidal activity, and is used as a miticide in orchards, nurseries and homes as well as against the red imported fire ant (Jin *et al.* 2009). Bifenthrin was first registered in the US and Europe in 1985 (European Commission 2012).

Permethrin (3-phenoxybenzyi *(+) cis, trans,* 3(2,2-dichlorovinyl)-2,2-dimethyicyclopropane carboxylate) is a type I pyrethroid, that is used in public health mosquito abatement programs, and on a variety of food stores and feed crops and livestock, in structures and buildings, including livestock housing and food-handling establishments. Permethrin is also used in numerous residential areas, both indoor and outdoor, and on pets and clothing. It was originally registered for use by the US EPA in 1979 (U.S.EPA 2006). Its use was banned in Europe in 2003 due to its toxicity to non-target organisms (European Commission 2000).

Cyfluthrin (3-(2,2-dichloroethenyl)-2,2-dimethylcyano(4-fluoro-3-phenoxyphenyl) methyl ester) is a type II pyrethroid that is often used in insecticidal sprays as well as on agricultural crops, stored products, public health situations (i.e., cockroaches, mosquitoes, and flies), and domestic pets. Its use was first approved in the US in 1987. In Europe its use was first approved in 1993, but its use was not extended in 2014 due to its toxicity to non-target organisms (European Commission 2014).

Lambda-cyhalothrin (3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethyl-cyano (3-phenoxyphenyl)methyl cyclopropane-carboxylate) is a type II pyrethroid, that is used on food and non-food crops, in greenhouses, in and around hospitals, for cattle (in ear tags), and in termite treatments (National Pesticide Telecommunications Network 2001). Residential use can be both indoors and outdoors on homes, ornamental plants, and lawns. Lambda-cyhalothrin was first registered for use in the US in 1988, and for use in Europe in 2002 (European Commission 2000).

Chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate) is a broad-spectrum organophosphate pesticide that is currently registered for use on food and non-food crops, golf course turf, industrial sites, greenhouse and nursery production, and wood products. It was first registered in the US in 1965. All homeowner use product registrations have been banned in the US in 2000 (Kelly and Patti 2011). In Europe, chlorpyrifos was first approved in 2006 (European Commission 2005).

The five insecticides have overlapping usage patterns, and are known to co-occur in surface water and sediment causing mixture effects on non-target organisms

## 1.4 Ecotoxicological Testing Using Invertebrate Species

### 1.4.1 The Importance of Aquatic Invertebrates in Ecotoxicological Testing

Aquatic invertebrates can be found in nearly any habitat from small temporary pools and small springs, to large lakes and rivers. Invertebrates are functionally important in aquatic ecosystems due to the ecosystem services they provide by accelerating detrital decomposition of organic matter, nutrient cycling, as well as the considerable food-source they represent for fishes, birds, and other aquatic organisms (Wilson 1992; van de Bund *et al.* 1994; Wallace and Webster 1996; Freckman *et al.* 1997; Chagnon *et al.* 2015).

Alterations in invertebrate abundance, physiology, and life history by pollutants can affect freshwater ecosystems, potentially impacting community composition and structure, loss of functional diversity and the ecological services that these communities provide (Samways 2005). Pollution by pesticides is widely recognized to be one of the major causes of the decline in biodiversity of aquatic invertebrates worldwide (Dudgeon *et al.* 2006; Geist 2011). In Europe, pesticide use has sharply reduced the regional biodiversity of stream invertebrates, such as mayflies and dragonflies, at pesticide concentrations that European regulations deem environmentally protective (Beketov *et al.* 2013). Thus, understanding toxic effects on invertebrates caused by contaminants is a first step in protecting aquatic ecosystems from bottom-up. Further, due to their small size, short reproduction cycle, and simple housing, invertebrates represent an ideal study organism to fill in the gaps in the field of mixture toxicity.

### 1.4.2 Main Study Species of this Research - *Chironomus dilutus* and *Hyalella azteca*

*Chironomus dilutus* (formerly known as *C. tentans*) and *Hyalella azteca* are commonly used ecotoxicological test species used in whole-sediment testing (e.g., Norberg-King *et al.* 2006; You *et al.* 2008; Weston *et al.* 2013), bioaccumulation studies (e.g., Bartlett *et al.* 2007; Franz *et al.* 2013), life-cycle assays (e.g., Norberg-King *et al.* 2006; Dussault *et al.* 2008), and ecotoxicological risk assessments (e.g., Harwood *et al.* 2009; Willming *et al.* 2013; Proulx and Hare 2014). In addition, a method for the use of *H. azteca* in whole-water testing was established in recent years (Brander *et al.* 2009; Werner *et al.* 2010; Deanovic *et al.* 2013) and has been the subject of recent gene expression studies in ecotoxicogenomics (Poynton *et al.* 2013).

*Chironomus dilutus* are chironomidae (non-biting midges), a family of nematoceran flies with global distribution (Figure 2).

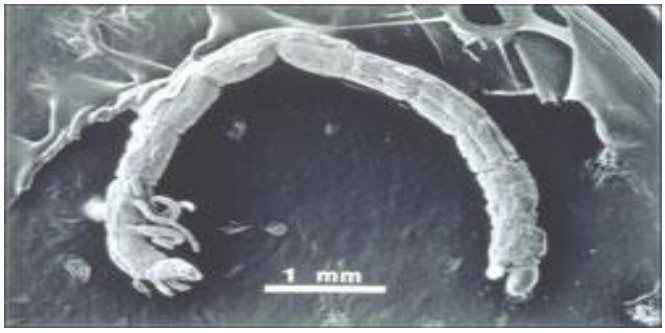


Figure 2 Chironomid larvae (*Photo credit: Dr. John Giesy, Michigan State University*)

Their life cycle consists of several aquatic larval stages that live in their protective tubes from two to seven weeks, depending on water temperature, and can reach up to 30 mm of body length before they eventually pupate while still in their tubes. After three days, pupae actively swim to the water surface and emerge in form of a winged adult several hours later. Adults mate in swarms soon after emerging and live for only three to five days as they do not feed. Their benthic larval stages represent an important fraction of the macro zoobenthos of most freshwater ecosystems. Many species of this family have adjusted to anoxic conditions and are dominant in polluted waters. Ecological differences among *Chironomus* species exist due to their feeding habits. Some *Chironomus* species, such as *C. dilutus*, filter particles from water overlying sediments (Walshe 1951), whereas others consume deposited sediment particles (Proulx and Hare 2014). Larvae feeding on deposited sediment can consume either oxic particles or anoxic sediments from below the oxic zone (Martin *et al.* 2008). Oxic particles are located either at the interface between the water column and the surface sediment or in the walls of burrows through which larvae pump oxygenated overlying water (Gallon *et al.* 2008). Insect-feeding birds, such as purple martins and swallows, as well as bats use chironomid pupae as food source. For many fish species, such as trout, banded killifish, and sticklebacks, but also for other aquatic invertebrates, such as predatory Hemipterans (true bugs) and Dytiscidae and Hydrophilidae (water beetles), both larvae and pupae represent an important food source (Ciborowski and Corkum 2003; Smits *et al.* 2005).

*Hyalella azteca* is a freshwater amphipod species commonly found in lakes, ponds, and streams throughout North America (Othman and Pascoe 2001; Figure 3).

Figure 3 Hyalella azteca *Photo credit: Barbara Albrecht (UC IPM Online)*

They belong to malacostracan crustaceans with no carapace and generally laterally compressed bodies. There are no larval stages as the eggs hatch directly into a juvenile form. *Hyalella azteca* develops through five pre-reproductive instars (juvenile stage) and an indefinite number of post-reproductive instars (Geisler 1944; Cooper 1965). Instars 6 and 7 represent the adolescent stage (when sexes can be differentiated) (Wade *et al.* 2004), while instar 8 corresponds to the nuptial stage (Geisler 1944; Cooper 1965; Pennak 1989). All later instars represent the adult stage (Pennak 1989). At 24 to 28°C, hatching can range from 5 to 10 days after fertilization (Cooper 1965) and amphipods can be expected to be in amplexus, in which a male grasps the female with its gnathopods while on the back of the female, first at about day 21 to 28 with release of the first brood between day 28 to 42 (Ingersoll *et al.* 1998).

As an epibenthic organism, *H. azteca* primarily live on the sediment surface and in algal mats. While their primary food source varies by habitat, they preferably feed on epiphytic algae, but also organic detritus and leaf litter (Wang *et al.* 2004). *H. azteca* represent an important food source for waterfowl such as White-winged Scoters and Lesser Scaup as well as several fish (e.g., Bluegill, Yellow Perch) and invertebrate species (e.g., Large Diving Beetle, Eastern Dobsonfly). The fact that *H. azteca* in the wild actually represents a species complex has resulted in differential contaminant sensitivity among species within the complex which could have implications for monitoring programs that rely on this organism (Duan *et al.* 2000; Weston *et al.* 2013).

## 1.5 Ecotoxicological Exposure Systems

### 1.5.1 Overview of Exposure Systems

Short-term, single-species laboratory tests provide efficient and economical exposures under controlled conditions to assess pesticide effects in a small space which many laboratories can afford (Stanley *et al.* 2005). In addition, laboratory exposures allow the use of a large number of replicates and different treatment concentrations and have the potential to be easily modified (e.g., exposure temperature or water quality parameters) as well as to study specific mechanistic endpoints for a species of interest (e.g., Connon *et al.* 2008; Weston *et al.* 2009). Multispecies aquatic toxicity tests represent a more realistic exposure scenario than single-species tests, as they allow to incorporate and modify a subset of parameters that are present in the environment (e.g., Kersting and Van Wijngaarden 1999; Sandberg and Landis 2001). The size of multispecies tests can range from 1-L mixed flask cultures (Stay and Jarvinen 1995; Sandberg and Landis 2001) to large-scale mesocosms of several thousands of liters. They can either be conducted under controlled lab conditions, for example in microcosm experiments, or in the field in form of mesocosms. Microcosms represent an increased ecological relevance compared to mixed flask cultures by providing information aquatic communities under controlled conditions on a bigger scale (Barry and Logan 1998). For example, Cuppen *et al.* (2002) used microcosms (glass aquaria) containing natural sediment in a climate room to study the effects of insecticide mixtures on introduced periphyton, zooplankton, macroinvertebrate species.

Large-scale toxicity studies on natural systems would represent the most realistic approach to study toxicity effects, but since this is not feasible due to the potential impact of such studies, mesocosms represent the most realistic exposure possible in multispecies testing while providing controlled chemical manipulations without impacting natural systems (Odum 1984). Such realistic exposure scenarios in the field can be used in ecological risk assessment, bridging the gap between simple single-species laboratory toxicity tests and field bioassessments, while still providing controls as well as replication that increases statistical power of the data (Stanley *et al.* 2005).

### 1.5.2 Definition of Aquatic Mesocosms

Aquatic mesocosms, or experimental water enclosures, are defined as a limited body of water with close to natural conditions, in which stressors of interest can be manipulated under controlled yet realistic environmental conditions such as diurnal temperature, UV light, and humidity changes. Mesocosms are a powerful approach to bridge laboratory exposures with field studies; providing a more realistic degree of ecological relevance while still allowing the use of replicates (Odum 1984).

Thus, mesocosm studies take into account physical, chemical, and ecological processes that are not possible to be studied in simpler laboratory tests (Giddings *et al.* 2001). Further, they allow for the study of aggregate responses of multiple species, biological compensation and recovery, ecosystem resilience, as well as the indirect effects on other trophic levels causing trophic cascade effects (Solomon 1996), without losing reliable reference conditions and replication. By integrating multiple direct and indirect effects up or down the food web, the responses obtained from mesocosm studies can be used in ecosystem and biogeochemical models.

### 1.5.3 Types and Use of Mesocosms

Mesocosms have been incorporated into the advanced stages of many current schemes for ecological risk assessment (Solomon 1996), and have been employed as research tools in both terrestrial and aquatic systems (Odum 1984; Farmer *et al.* 1995; Ahn and Mitsch 2002) at both small (Chambers 1992; Pendleton *et al.* 2014; Shibata *et al.* 2014) and large spatial scales (Van Geest *et al.* 1999; Sorf *et al.* 2014). According to Newman (1995) there are three different types of experimental units to investigate community structure and function: microcosm, mesocosm, and natural water bodies. In the field of ecotoxicology, microcosms are laboratory systems that simulate a part of or an entire ecosystem. Mesocosms are definite outdoor systems of a certain size, and thus bridge the gap between microcosm and natural water bodies. Crossland *et al.* (1993) define a mesocosm by size and therefore consider any system bigger than 15 $m^3$ as a mesocosm.

Mesocosm experiments have some inherent limitations that should be considered. Small-scale mesocosm experiments may have reduced ecological complexity relative to a larger ecosystem such

as a pond or lake (Ahn and Mitsch 2002). Also, enclosure size and experimental duration can affect results from mesocosm experiments (Gehart and Likens 1975, Stephenson et al. 1984). Huston (1999) noted that neither large- nor small-scale mesocosm experiments can solely address the wide range of processes at work in large scale ecosystems, but rather each type of experiment has its niche in the process of facilitating a better understanding of ecological processes across the landscape.

The most discussed limitation of mesocosm experiments are the occurrence of artificial conditions, termed ‘‘chamber’’ or ‘‘wall effects’’ (Carpenter 1996, MacNally 1997, Schindler 1998, Gry et al. 1999). Chamber effects often include decreased $O_2$ concentrations, altered water turbidity, pH, nutrient supply, increased temperatures, and microbial growth on enclosure walls. These chamber effects and temporal concerns can be alleviated by monitoring conditions within mesocosms and comparing them with unenclosed, ambient reference sites.

### 1.6 Objectives

Given the increased use of insecticides and their alarming effects on non-target organisms of aquatic ecosystems and food webs, understanding the effects of complex mixtures of contaminants is one of the major challenges that the field of ecotoxicology is currently facing. The protection goals of legislation and regulatory authorities include populations, communities, and ecosystems. Thus, integrative studies are necessary to ensure that toxicological assessments are effective in aiding ecosystem management efforts. Invertebrates are of special interest not only because they represent an essential component in the food web, and avoid the use of vertebrates in ecotoxicological testing, but also because they share similarities in biochemical pathways with humans; e.g., *Daphnia pulex* has recently been recognized as a National Institutes of Health (NIH) research organism (Colbourne *et al.* 2011).

To improve the understanding of pesticide mixture effects, this project exemplarily integrated single-species laboratory toxicity tests with the use of field-based, multiple-species mesocosms for assessing contaminant mixture effects using the examples of commonly used insecticides.

The primary objectives of this research were:

1) To compare the effectiveness of *C. dilutus* and *H. azteca* toxicity tests and two different sublethal endpoints, growth and motility to detect toxicity caused by four current-use insecticides: three pyrethroids; bifenthrin, permethrin, and cyfluthrin, and one organophosphate; chlorpyrifos (Chapter 2). Hypothesis 1.1: Motility and growth are sensitive endpoints that will respond to environmentally relevant concentrations of pesticides, which sometimes can be below the limit of detection of current-use analytical methods. Hypothesis 1.2 The sublethal effect of pyrethroids will be greatest on *H. azteca*, while that of organophosphates will be greatest on *C. dilutus* due to the differences in sensitivity among the two species.

2) To evaluate effects of tertiary mixtures on *C. dilutus* and the effectiveness of sublethal endpoints in assessing mixture toxicity testing for regulatory applications and monitoring studies (Chapter 3). Hypothesis 2.1: Exposure of *C. dilutus* to a tertiary mixture of insecticides with different mode of action will cause a greater negative response than an exposure to each compound individually. Hypothesis 2.2: The sensitivity of sublethal endpoints will allow for biologically significant impact assessments resulting from low-level mixture concentration exposures.

3) To monitor the long-term contaminant mixture effects on the invertebrate community as well as the fate of the contaminants in both the water column and the sediment using outdoor mesocosms (Chapter 4). Hypothesis 3.1: The contaminant mixtures will significantly affect the biomass, structure and function of invertebrate community at environmentally relevant concentrations. Hypothesis 3.2: The pyrethroids will dissipate from the water column more quickly than the organophosphate.

# 2. A comparison of the Sublethal and Lethal Toxicity of Four Pesticides in *Hyalella azteca* and *Chironomus dilutus*

*A similar version of this chapter was published as: Simone Hasenbein, Richard E. Connon, Sharon P. Lawler, Juergen Geist (2015). Environmental Science and Pollution Research, DOI: 10.1007/s11356-015-4374-1*

## 2.1 Abstract

Laboratory toxicity testing is the primary tool used for surface water environmental risk assessment, however there are critical information gaps regarding the sublethal effects of pesticides. In 10-day exposures, we assessed the lethal and sublethal (motility and growth) toxicities of four commonly used pesticides, bifenthrin, permethrin, cyfluthrin, and chlorpyrifos, on two freshwater invertebrates, *Chironomus dilutus* and *Hyalella azteca*. Pyrethroids were more toxic than the organophosphate chlorpyrifos in both species. Bifenthrin was most toxic to *H. azteca* survival and growth and cyfluthrin was most toxic to *C. dilutus*. However, cyfluthrin had the greatest effect on motility on both *H. azteca* and *C. dilutus*. The evaluated concentrations of chlorpyrifos did not affect *C. dilutus* motility nor growth, but significantly impacted *H. azteca* growth. Motility served as the most sensitive endpoint in assessing sublethal effects at low concentrations for both species, while growth was a good indicator of toxicity for all four pesticides for *H. azteca*. The integration of sublethal endpoints in ambient water monitoring and pesticide regulation efforts could improve identification of low-level pesticide concentrations that may eventually cause negative effects on food webs and community structure in aquatic environments.

## 2.2 Introduction

Contaminants such as pesticides can pose major threats to freshwater biodiversity (Dudgeon *et al.* 2006; Geist 2011; Connon *et al.* 2012), as aquatic ecosystems worldwide are "sinks" for contaminants discharged from areas of intense pesticide use (Scholz *et al.* 2012). Insecticides such as pyrethroids and organophosphates are of particular concern due to their broad-spectrum aquatic toxicities (Ankley and Collyard 1995). They are highly toxic to non-target organisms such as fish and aquatic invertebrates (Clark and Matsumura 1982; Werner and Moran 2008). Many current-use insecticides are neurotoxic compounds, which exert sublethal effects on aquatic organisms that can lead to severe health or reproductive impairment (Rakotondravelo *et al.* 2006; Johnson *et al.* 2008; Connon *et al.* 2012). Pyrethroids are known to inhibit sodium channels in the axonal membranes of nerve cells (Clark and Matsumura 1982), while organophosphates competitively inhibit the enzyme acetylcholinesterase in nerve synapses (Karnak and Collins 1974; Wheelock *et al.* 2005). Depending on exposure concentration, both pesticide classes result in hyperactivity and eventual failure of the nervous system (Haya 1989; Werner and Moran 2008). While acute toxicity to fish and aquatic invertebrates is rare, sublethal effects on key prey species eventually affecting food webs are of greatest concern (Brooks *et al.* 2012; Scholz *et al.* 2012).

Sublethal responses such as swimming impairment and growth are suitable endpoints for evaluating organism fitness since they integrate biochemical and physiological processes and have been shown to be highly sensitive biomarkers for low-level pesticide concentrations (Christensen *et al.* 2005; Geist *et al.* 2007; Beggel *et al.* 2010). However, these sublethal endpoints are not necessarily integrated in ambient water monitoring or regulatory toxicity assessments. While growth is a relatively common toxicity endpoint in fish studies, it is rarely used for invertebrates. Impaired swimming ability is generally not quantified as an endpoint in standard toxicity testing methods despite its obvious importance for the ecological fitness of a species (Christensen *et al.* 2005; Floyd *et al.* 2008; Weston and Lydy 2010). Thus, there is a pressing need for validating the effectiveness of these sublethal endpoints, if such endpoints are to be integrated in detecting water toxicity in water monitoring and regulatory toxicity assessments.

In this study we compared the lethal and sublethal toxic effects of two commonly-used type-I pyrethroids (bifenthrin and permethrin), one type-II pyrethroid (cyfluthrin), and the organophosphate chlorpyrifos on *Chironomus dilutus* larvae and *Hyalella azteca*. These pesticides were selected based on their prevalence in the environment and their relative toxicities to non-target species (Hintzen *et al.* 2009; Bereswill *et al.* 2013; Li *et al.* 2013). In a recent study on pyrethroids encompassing 25 states across the U.S., bifenthrin was the most frequently detected (58% of samples), followed by permethrin (31%) and cyfluthrin (14%) (Hladik and Kuivila 2012). A study analyzing water samples from California creeks detected chlorpyrifos at concentrations between 11.8 and 1,874 ng/L (Anderson *et al.* 2014). All four pesticides are used for similar pest treatments such as for agriculture and landscape maintenance, and were regularly detected in the same water or sediment samples in recent studies (Weston *et al.* 2008; Budd *et al.* 2009; Weston *et al.* 2013). The selected pesticides are all neurotoxins with different neurological target sites and/or modes of action. The two types of pyrethroids cause toxicity through similar modulations of the voltage-gated sodium channels, but the degree of modification of sodium currents is different; single sodium channel currents are prolonged to a greater extent with type II than type I pyrethroids (Wouters and van den Bercken 1978; Clark and Matsumura 1982; Nasuti *et al.* 2003). Organophosphates (e.g., chlorpyrifos) inhibit acetylcholine esterase activity (Malison *et al.* 2010; Hua *et al.* 2013) directly impacting the synaptic signal. Varying modes of action could thus drive various exposure effects among the different test endpoints selected.

*C. dilutus* larvae and *H. azteca* are often used in toxicity testing because of their high sensitivity to pyrethroids and organophosphates (Ankley *et al.* 1994; Rakotondravelo *et al.* 2006; Deanovic *et al.* 2013; Weston *et al.* 2014). Both species are highly relevant for environmental risk assessments as they are found in water bodies throughout the Americas and are important food sources for fish, amphibians, aquatic insects, and other organisms. Both species were selected for this study because they reflect differences in habitat that may result in different exposure to contaminants. The larval stage of *C. dilutus* is an endobenthic deposit feeder, where it uses the sediment and debris to build protective cases (Ankley *et al.* 1994; Lydy and Austin 2004; Ding *et al.* 2011). *H. azteca* is an epibenthic detritivore, often found on macrophytes and other surfaces, and periodically moves into the water column. In addition to its use in sediment testing, *H. azteca* is also listed as a supplemental

species for water column analyses in the U.S. Environmental Protection Agency whole effluent toxicity testing guidance (U.S.EPA 2002).

The aim of this study was to compare the effectiveness of the *C. dilutus* and *H. azteca* tests to detect toxicity caused by four current-use insecticides: three pyrethroids, bifenthrin, permethrin, and cyfluthrin, and one organophosphate, chlorpyrifos. In addition, we evaluated the use of two different sublethal endpoints, growth and motility, in detecting low-level insecticide concentrations.

## 2.3 Materials and Methods

*Test Organisms*

*Chironomus dilutus* (second instar larvae, 10-12 days old) were obtained from Aquatic Biosystems (Fort Collins, CO, USA) and *H. azteca* (7-10 days old) from Aquatic Research Organisms (Hampton, NH, USA). Upon arrival, animals were transferred to aerated 7-L aquaria and acclimated to laboratory test conditions for 48h. During the acclimation period, approximately 50% of the transport water was changed twice daily and refilled with test control water, i.e., deionized water modified to attain U.S. EPA moderately hard specifications (hardness 90-100 mg/L $CaCO_3$, alkalinity 50-70 mg/L as $CaCO_3$, SC 330-360 µS/cm and pH 7.8-8.2) (Eide and Johansson 1994; U.S.EPA 2002). Once a day, *C. dilutus* and *H. azteca* were fed 10 ml of 4 g/L Tetramin slurry (Tetra®) and 20 ml of YCT (yeast-cerophyll-trout chow), respectively.

*Exposure Assessments*

Ten-day (10d) toxicity tests with *C. dilutus* and *H. azteca* were conducted in a temperature-controlled room at 23 ± 2ºC with a 12:12h dark:light photoperiod. Bifenthrin (CAS# 82657-04-3, purity >98%), permethrin (CAS# 52645-53-1, purity >95.7%), cyfluthrin (CAS# 68359-37-5, purity >99%), and chlorpyrifos (CAS# 5598-13-0, purity >99.5%) were purchased from Chem Service (West Chester, PA, USA). Pesticide-grade methanol was used as a solvent carrier for the pesticide treatments, and in solvent controls, to a final concentration of 0.01% in exposure water. Corresponding stock solutions were spiked into control water according to target concentrations, and

mixed thoroughly. Organisms were randomly added to each replicate beaker. In total, organisms were exposed to a geometric progression of seven concentrations of each pesticide (Table 1) determined from preliminary 10d toxicity test data (not reported), a solvent control, and a negative control. At test initiation and after each water renewal, organisms were fed 1.5 ml of 4 g/L Tetramin slurry (Tetra®) for *C. dilutus* and 1 ml of YCT for *H. azteca*.

The 10d toxicity tests were based on U.S. EPA protocols for static sediment toxicity testing (U.S.EPA 2000), with the following modifications for each species. For *C. dilutus*, four replicate 1 L glass beakers, each containing a substrate of 20 g silica sand that was clean and baked (four hours at 450°C), 750 ml of treatment water, and 10 organisms. The *H. azteca* 10d toxicity tests were modified for water column exposures, as described in the Quality Assurance Management Plan for the State of California's Surface Water Ambient Monitoring Program (SWAMP 2002). Briefly, each concentration tested included four replicate 250 ml glass beakers, each containing 100 ml of treatment water, 10 organisms and a 2 $cm^2$ piece of Nitex® screen as artificial substrate.

Mortality was recorded daily and any dead organisms were removed from the test vessels. In addition, 70% of each test solution was renewed at 24h (*C. dilutus)* or 48h (*H. azteca*) time intervals, based on similar studies on *C. dilutus* (Xu *et al.* 2007) and *H. azteca* (Deanovic *et al.* 2013). At the time of water renewal, debris was removed and water quality parameters [pH, specific conductance (SC), dissolved oxygen (DO), temperature (T)] of renewal and wastewater were measured. Test vessels were randomly distributed after each water renewal.

To evaluate movement and activity of organisms at test termination, swimming behavior was measured as motility in cm/s. Both species are generally sedentary, but are inclined to swim when they are not provided substrate. Therefore, surviving organisms were transferred individually into corresponding filming chambers; a 5.5 cm (*C. dilutus*) or 1.3 cm (*H. azteca*) diameter well in a five-welled white PVC plate containing water from the respective beaker in which they were exposed. *C. dilutus* larvae had to be carefully teased from their cases before being transferred. To improve lighting quality and contrast of the videos, the white PVC plate was then placed on a light board. Video settings and plate position were adjusted to achieve a standardized focus point for each recording. Videos were recorded in MPEG-2 format, using a Panasonic® black and white CCTV camera (12V

DC) filming all five filming chambers from the top. The camera was connected to a portable laptop-computer via a USB Frame grabber (Model WinTV-HVR 950, Hauppauge Computer Works, Hauppauge, NY). Thirty frames per second were collected for each organism, over a period of 80 seconds. Recorded videos were then analyzed using the Ethovision XT 6.1 Software (Noldus Information Technology Inc., Leesburg, VA) to determine motility (cm/s). The two-dimensional movement tracks were analyzed by measuring the movement of the center-point of each organism's body. While *H. azteca* move rectilinearly, *C. dilutus* display an undulating movement, resulting in a greater calculated motility than for *H. azteca*.

Following video recording, the organisms were transferred from the filming chambers onto individual pre-weighed tin dishes (pooled per treatment replicate), desiccated at 60°C following methods described by Nahon et al. (2010), and weighed using a Mettler® Toledo AL104 balance (0.1 mg accuracy). To examine 10d growth (increase of weight in grams over time), the weights of five subsamples of ten organisms were measured at test initiation and compared to the weights of surviving individuals at test termination. Due to limited scale sensitivity, organisms were pooled per replicate beaker, and only treatment replicates with five or more surviving individuals are reported herein. Mean individual dry weight in milligrams was calculated for each replicate for statistical analysis. The calculated 10d growth was compared between treatments and controls to determine pesticide effects.

*Analytical Chemistry*

At test initiation, 1-L water samples for each treatment and the solvent control were collected, and stored in amber glass bottles in the dark at 4°C for subsequent chemical analyses (Table 2).

Table 2 Nominal and measured concentrations (ng/L) for bifenthrin, permethrin, cyfluthrin, and chlorpyrifos used in 10-day exposures to *C. dilutus* and *H. azteca*

| | Pesticide Concentration (ng/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Bifenthrin | | Permethrin | | Cyfluthrin | | Chlorpyrifos | |
| | *Nominal* | *Measured* | *Nominal* | *Measured* | *Nominal* | *Measured* | *Nominal* | *Measured* |
| *C. dilutus* | 15.00 | 10.75 | 15.00 | 16.31 | 2.00 | 2.47 | 80.00 | 53.54 |
| | 29.10 | 18.57 | 29.10 | 24.77 | 4.11 | 3.59 | 131.80 | 91.16 |
| | 56.46 | 41.60 | 56.46 | 44.98 | 8.43 | 9.05 | 217.15 | 203.87 |
| | 109.54 | 94.41 | 109.54 | 104.60 | 17.32 | 11.93 | 357.77 | 274.19 |
| | 212.53 | 169.31 | 212.53 | 209.36 | 35.57 | 25.15 | 589.45 | 397.96 |
| | 412.34 | 378.82 | 412.34 | 310.74 | 73.04 | 63.55 | 971.14 | 632.57 |
| | 800.00 | 552.60 | 800.00 | 735.40 | 150.00 | 123.51 | 1600.00 | 1166.53 |
| *H. azteca* | 1.00 | 0.98 | 5.00 | 4.98 | 0.20 | <LOD | 10.00 | 8.33 |
| | 1.59 | 1.33 | 8.24 | 8.53 | 0.38 | <LOD | 17.63 | 12.20 |
| | 2.52 | 2.23 | 13.57 | 13.05 | 0.74 | <LOD | 31.07 | 24.48 |
| | 4.00 | 4.08 | 22.36 | 19.30 | 1.41 | 1.98 | 54.77 | 31.31 |
| | 6.35 | 5.92 | 36.84 | 34.22 | 2.71 | 2.95 | 96.55 | 65.65 |
| | 10.08 | 9.48 | 60.70 | 58.97 | 5.21 | 4.64 | 170.19 | 93.77 |
| | 16.00 | 15.08 | 100.00 | 93.66 | 10.00 | 6.62 | 300.00 | 239.46 |

< LOD indicates cyfluthrin concentration was below limit of detection, but concentrations were estimated by using the average factor between each available measured concentration (0.66) resulting in the following concentrations: 0.59, 0.89, 1.33 ng/L. This data was included in the statistical analysis

Within 48h, samples were spiked with trans-permethrin (dimethyl D6, EQ Laboratories, Atlanta, GA, USA) as a recovery surrogate and extracted using solid phase extraction cartridges (Supelclean ENVI™ - C18, 500 mg, Sigma-Aldrich, St. Louis, MO, USA). Cartridges were pre-conditioned using 12 mL 1:1 ethyl acetate:hexane, 12 mL methanol, and 12 mL MilliQ water (Millipore). Samples were loaded on the cartridge and eluted with 10 mL 1:1 ethyl acetate:hexane and evaporated to 0.4 mL at 40° under a gentle stream of nitrogen. As an internal standard, 4-4' dibromo-octafluorobiphenyl (Chem Service, West Chester, PA, USA) was added (Parry and Young 2013). Extracts were analyzed using an HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an HP-5973N quadrupole mass spectrometer detector operated in electron capture negative ionization mode (GC-ECNI-MS) with methane as the reagent gas (Hladik and Kuivila 2012; Weston *et al.* 2013). The

gas chromatograph was equipped with a Supelco DB-5MS (30 m x 0.25 mm with a 0.25 µm film thickness) with Helium as the carrier gas. A 1-µL of sample was injected in splitless mode (injector temperature 280°C, purge time 1.5 min). Instrumental calibration was performed using nine sets of calibration standard solutions containing all four pesticides (each purchased as 100 µg/ml solution in acetonitrile, Chem Service, West Chester, PA, USA), the surrogate trans-permethrin (dimethyl D6), and the internal standard 4-4' dibromo-octafluorobiphenyl in hexane. Quantification of the pesticides was based on peak areas and comparing them with a calibration curve normalized to the internal standard response. All calibration curves had an $r^2 > 0.99$. Quality-assurance/quality-control was conducted by analyzing a method blank of deionized water (Milli-Q) to ensure that no contamination occurred during sampling extraction and analysis, and by analyzing two laboratory spike samples to determine whether the sample matrix contributes bias to the analytical results and to what degree the method is successful in recovering the target analytes. The surrogate trans-permethrin was added to each sample, including the blank, before extraction to monitor matrix effects and overall method performance. Surrogate recoveries were on average 111.21% with a range between 102.01 – 116.59% confirming high extraction efficiency. Reported values were not corrected for surrogate recovery. 4-4' dibromo-octafluorobiphenyl was added to sample extracts before analysis in order to correct quantitative differences in extract volume as well as to monitor instrument conditions. Instrumental limit of detections (whole water) were as follows: 0.6 ng/L bifenthrin, 4.8 ng/L permethrin, 1.4 ng/L cyfluthrin, and 0.8 ng/L chlorpyrifos.

No pesticides were detected in the controls or the method blank. In particular, average recoveries for bifenthrin were 84.97% (range 63.81 – 102.00%), for permethrin 93.15% (range 75.36 – 108.73%), for cyfluthrin 93.79% (range 66.20 – 140.43%), and for chlorpyrifos 71.68% (range 55.10 – 93.88%). Pesticide concentrations are herein reported as measured concentrations. For cyfluthrin (exposure to *H. azteca*), three treatments were below the limit of detection. To include these treatments for statistical analysis, the concentrations were estimated by using the average factor between measured concentrations (0.66), resulting in the following concentrations: 0.59 ng/L, 0.89 ng/L, and 1.33 ng/L.

*Statistical Analysis*

No observed effect concentrations (NOEC) were determined using one-way ANOVA followed by a Dunnett's multiple comparison. Where data were not normally distributed, but homogeneity of variances was met, a Kruskal-Wallis test was applied. Shapiro-Wilk test and Levene's test were used to test normality and equality of variances, respectively. All differences discussed below are significant unless otherwise noted. All analyses were carried out using Minitab 17 Statistical Software 2013 (Minitab, Inc., State College, PA, USA) with a significance level at $\alpha = 0.05$.

Concentrations that caused a 50% reduction in survival ($LC_{50}$) and sublethal endpoints ($EC_{50}$) were determined by fitting non-linear regression curves to the measured toxicity data using the DRC package in the program R, version 2.3-96 (Ritz and Streibig 2005; R Core Team 2013). For all data, log-normal, log–logistic and Weibull functions were fitted with the optimal model fit chosen for each dataset by the distribution that had the lowest Akaike's information criterion value. The optimal model was confirmed by a goodness of fit test.

## 2.4 Results

*Water Quality Parameters*

Water quality parameters remained stable throughout all exposures. Ranges for *C. dilutus* tests were as follows: 7.5 – 8.6 pH, 242.7 – 290.7 µS/cm SC, 4.3 – 9.4 mg/L DO, and 20.2 – 22.7°C T, and for *H. azteca*: 7.6 – 8.5 pH, 257.4 – 296.3 µS/cm SC, 4.9 – 9.7 mg/L DO, and 20.9 – 22.8°C T. Mean control survival of *C. dilutus* and *H. azteca* was 98% (SE = ±0.03) and 100% (SE = ±0.00), respectively, meeting test acceptance criteria for these species (U.S.EPA 2000; SWAMP 2002).

*Effects on survival*

Cyfluthrin was the most toxic pesticide to *C. dilutus* with an $LC_{50}$ of 17.36 ng/L, followed by bifenthrin (101.07 ng/L), permethrin (166.80 ng/L) and chlorpyrifos (335.20 ng/L) (Figure 4a and Table 3).

The lowest $NOEC_{Survival}$ was also greatest for cyfluthrin (9.05 ng/L), followed by bifenthrin (41.60

ng/L), permethrin (44.98 ng/L), and chlorpyrifos (203.87 ng/L).

Survival of *H. azteca* was most sensitive to bifenthrin ($LC_{50}$ = 2.01 ng/L), followed by cyfluthrin (2.89 ng/L), permethrin (40.90 ng/L), and chlorpyrifos (58.41 ng/L) (Figure 4b and

Table 3). The $NOEC_{Survival}$ of cyfluthrin and bifenthrin were 1.33 ng/L, for permethrin 19.30 ng/L, and for chlorpyrifos 31.31 ng/L.

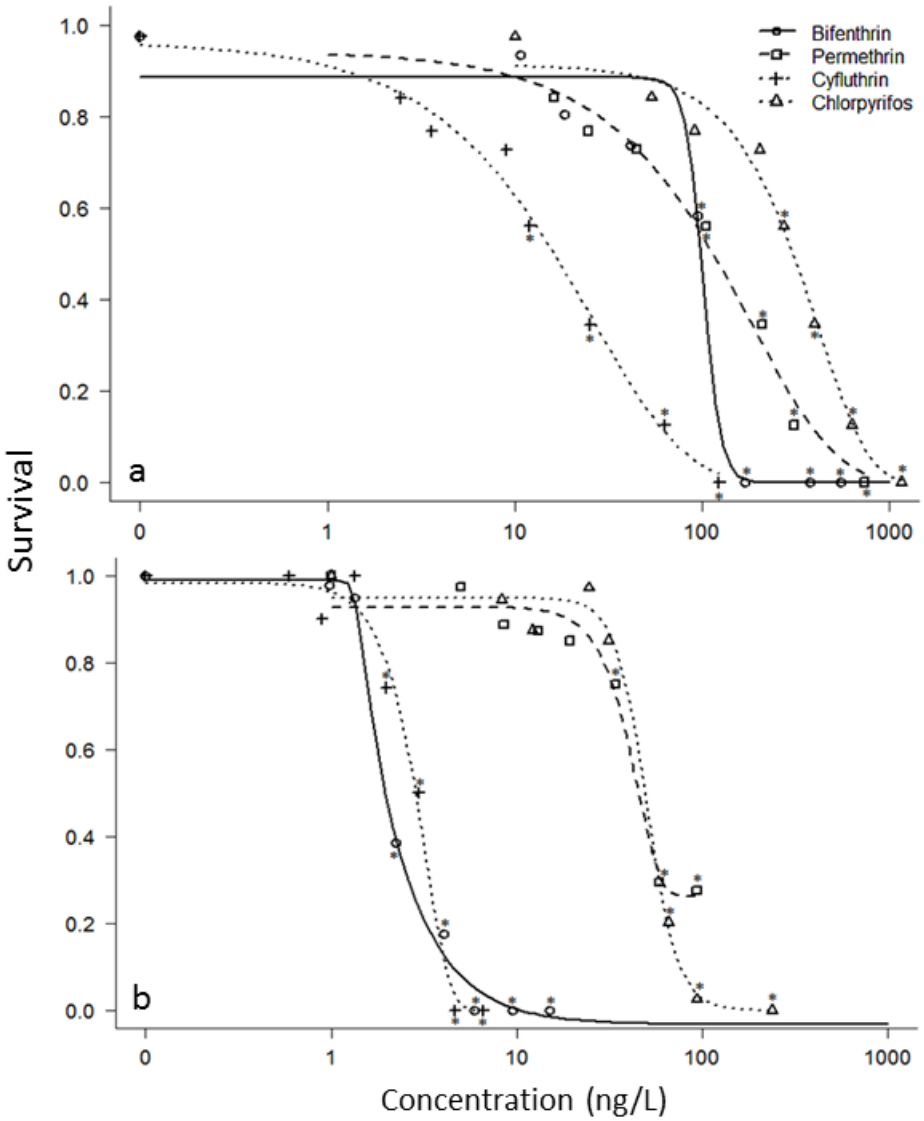


Figure 4 Lethal effects of bifenthrin, permethrin, cyfluthrin, and chlorpyrifos to *C. dilutus* (a) and *H. azteca* (b). Specific dose-response models (log-logistic or Weibull) were fitted to survival data for both species using the "mselect" function in the "drc" package. Y-axis = Survival. X-axis = concentration (ng/L) for each pesticide. Asterisks indicate significant differences compared to the control ($p < 0.05$)

Table 3 Effect concentrations calculated for 10-day exposures of *C. dilutus* and *H. azteca* to bifenthrin (BIF), permethrin (PERM), cyfluthrin (CYF), and chlorpyrifos (CLF)

| | | Effect concentration (ng/L) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Chemical | NOEC Survival | LC50 | SE | 95% C.I. | NOEC Motility | EC50 Motility | SE | 95% C.I. | NOEC Weight | EC50 Weight | SE | 95% C.I. |
| *C. dilutus* | BIF | 41.60 | 101.07 | 32.33 | 34.95 – 167.20 | 41.60 | 52.67 | 29.39 | 1.01 – 114.96 | < 10.75 | 15.08 | 3.50 | 7.70 – 22.44 |
| | PERM | 44.98 | 126.16 | 23.43 | 78.23 – 174.91 | 24.77 | 33.96 | 17.57 | 11.05 – 56.85 | 16.31 | 26.81 | 4.71 | 16.97 – 36.63 |
| | CYF | 9.05 | 17.36 | 3.04 | 11.15 – 23.57 | 3.59 | 4.81 | 2.46 | 0.05 – 57.64 | 3.59 | 14.48 | 5.84 | 2.15 – 26.80 |
| | CLF | 203.87 | 335.20 | 34.52 | 264.59 – 405.80 | --- | --- | --- | --- | --- | --- | --- | --- |
| *H. azteca* | BIF | 1.33 | 2.01 | 0.10 | 1.71 – 2.30 | < 0.98 | 1.40 | 0.27 | 0.81 – 1.99 | 0.98 | 1.65 | 0.22 | 1.18 – 2.12 |
| | PERM | 19.30 | 40.90 | 3.26 | 34.22 – 47.56 | 4.98 | 38.63 | 11.61 | 14.88 – 62.37 | < 4.98 | 4.03 | 0.85 | 2.25 – 5.80 |
| | CYF* | 1.33 | 2.89 | 0.13 | 2.61 – 3.16 | 0.59 | 0.53 | 0.13 | 0.26 – 0.79 | 0.59 | 1.19 | 0.10 | 0.96 – 1.40 |
| | CLF | 31.31 | 50.41 | 4.93 | 40.32 – 60.50 | < 8.33 | --- | --- | --- | 12.20 | 25.08 | 10.23 | 3.48 – 46.67 |

NOEC = no observed effective concentration, LC50 = lethal concentration resulting in 50% mortality of the population, EC50 = effect concentration resulting in 50% reduction in growth, SE = standard error, C.I. = Confidence Interval, '---' indicates value was not calculable because the levels of response did not amount to 50% relative to the control ($EC_{50}$ values) or did not cause significant effects (NOEC). * indicates the determined effect concentrations are based on a calculation that includes estimated concentrations (see table 1)

*Effects on Motility*

Average control motility of *C. dilutus* was 1.88 cm/s (SE ±0.25). Exposure to all three pyrethroids caused a decrease in motility of *C. dilutus*, while chlorpyrifos did not affect this endpoint (Figure 5a).

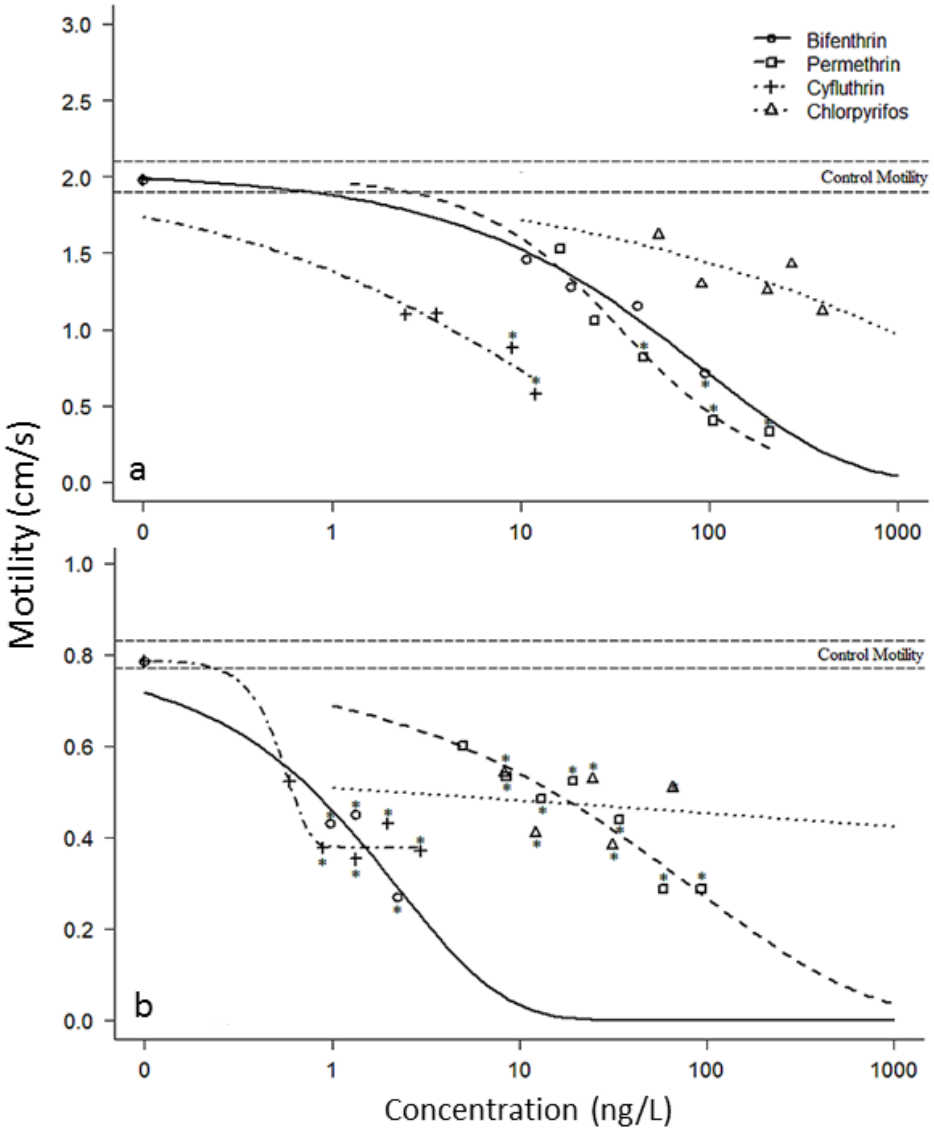


Figure 5 Sublethal effects of bifenthrin, permethrin, cyfluthrin, and chlorpyrifos on motility of *C. dilutus* (a) and *H. azteca* (b). Specific dose-response models (log-logistic or Weibull) were fitted to motility data for both species using the "mselect" function in the "drc" package. Y-axis = Motility (cm/s). X-axis = concentration (ng/L) for each pesticide. Asterisks indicate significant differences compared to the control ($p < 0.05$)

At the lowest concentrations causing a significant effect, bifenthrin was most potent in reducing the motility by 62% to 0.72 cm/s (SE ±0.24) at 94.41 ng/L followed by permethrin and cyfluthrin which reduced motility by 56% to 0.82 cm/s (SE ±0.09) at 44.98 ng/L and by 53% to 0.88 cm/s (SE

±0.17) at 9.05 ng/L, respectively. Cyfluthrin was the most toxic pyrethroid affecting *C. dilutus* motility at an $EC_{50}$ of 4.81 ng/L, followed by permethrin (44.59 ng/L) and bifenthrin (52.67 ng/L) (

Table 3). The lowest $NOEC_{Motility}$ was determined for cyfluthrin (3.59 ng/L), followed by permethrin (24.77 ng/L), and bifenthrin (41.60 ng/L). Average control motility of *H. azteca* was 0.56 cm/s (SE ±0.05). Exposure to all three pyrethroids caused a decrease in motility of *H. azteca*, however, no effect of chlorpyrifos on motility was observed (Figure 5b). At the lowest concentrations causing a significant effect, cyfluthrin was most potent in reducing the motility by 32% to 0.38 cm/s (SE ±0.08) at 0.89 ng/L followed by bifenthrin and permethrin which reduced motility by 23% to 0.43 cm/s (SE ±0.06) at 0.98 ng/L and 0.53 cm/s (SE ±0.03) at 8.53 ng/L, respectively. Cyfluthrin was the most toxic pyrethroid on *H. azteca* motility ($EC_{50}$ = 0.53 ng/L), followed by bifenthrin (1.40 ng/L) and permethrin (38.63 ng/L) (Table 3b). The lowest $NOEC_{Motility}$ was determined for cyfluthrin (0.59 ng/L), followed by bifenthrin (< 0.98 ng/L), permethrin (4.98 ng/L), and chlorpyrifos (< 8.33 ng/L). The $NOEC_{Motility}$ of *H. azteca* for cyfluthrin (0.59 ng/L) was higher than the $EC_{50}$ value (0.53 ng/L) due to the use of an estimated concentration rather than the measured concentration which was below the limit of detection.

*Effects on Growth*

Average initial dry weight of *C. dilutus* at test initiation was 0.17 mg (SE = ±0.01) per individual compared to an average final 10d dry weight of 1.55 mg (SE = ±0.05) per individual in the controls. These results indicate an average growth that was 9.12 times the initial weight over the 10d test period. All pyrethroids significantly affected growth of *C. dilutus*, while exposure to the organophosphate did not cause any effect (Figure 6a).

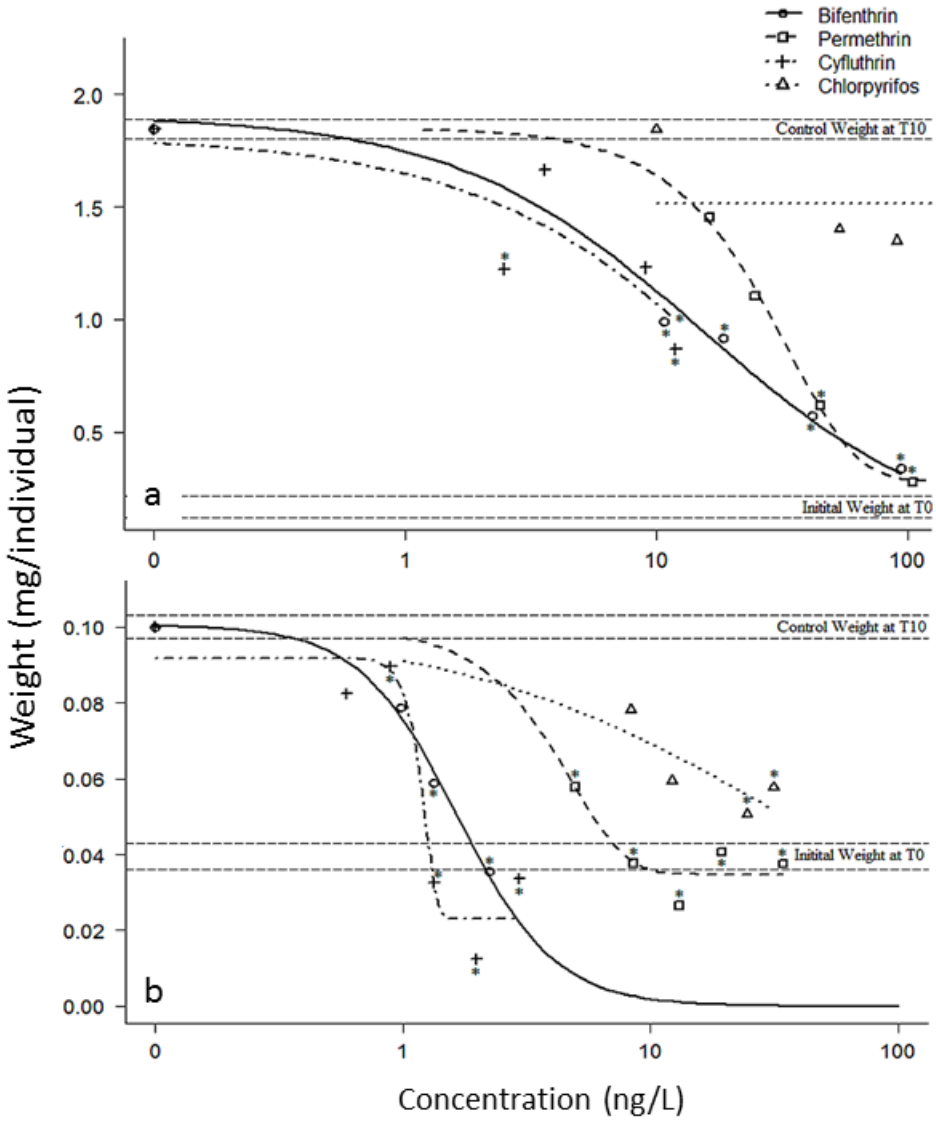


Figure 6 Sublethal effects of bifenthrin, permethrin, cyfluthrin, and chlorpyrifos on weight of *C. dilutus* (a) and *H. azteca* (b). Specific dose-response models (log-logistic or Weibull) were fitted to weight data for both species using the "mselect" function in the "drc" package. Y-axis = Final weight (mg/surviving individuals). X-axis = concentration (ng/L) for each pesticide. Asterisks indicate significant differences compared to the control ($p < 0.05$)

At the lowest concentration causing a significant effect, bifenthrin was most potent in growth inhibition reducing weight by 36% to 0.99 mg (SE ±0.13) per individual at 10.75 ng/L, followed by permethrin and cyfluthrin which reduced weight by 29% to 1.11 mg (SE ±0.23) per individual at 24.77 ng/L and by 21% to 1.23 mg (SE ±0.14) per individual at 9.05 ng/L, respectively. Cyfluthrin was the most toxic pyrethroid affecting *C. dilutus* weight ($EC_{50}$ = 14.48 ng/L), followed by bifenthrin (15.08 ng/L) and permethrin (26.81 ng/L) (Figure 6b). The NOECWeight for cyfluthrin was 3.59 ng/L, followed by bifenthrin (< 10.75 ng/L) and permethrin (16.31 ng/L).

All four pesticides significantly affected growth of *H. azteca* (Fig. 3b). Average initial dry weight

of a subsample of *H. azteca* at test initiation was 0.040 mg (SE = ±0.004) per individual compared to an average final 10d dry weight of 0.100 mg (SE = ±0.000) per individual in the controls. These results indicate an average growth of 2.50 times the initial weight over the 10d test period. At the lowest concentration causing a significant effect, chlorpyrifos was most potent in growth inhibition reducing weight by 49% to 0.051 mg (SE ±0.014) per individual at 24.48 ng/L followed by permethrin, bifenthrin, and cyfluthrin which reduced weight by 42% to 0.058 mg (SE ±0.006) per individual at 4.98 ng/L, by 41% to 0.059 mg (SE ±0.012) per individual at 1.33 ng/L, and by 11% to 0.089 mg (SE ±0.006) per individual at 0.89 ng/L, respectively. Cyfluthrin was the most toxic pesticide on *H. azteca* weight ($EC_{50}$ = 1.19 ng/L), followed by bifenthrin (1.65 ng/L), permethrin (4.03 ng/L), and chlorpyrifos (25.08 ng/L) (Table 3b). The lowest $NOEC_{Weight}$ was determined for cyfluthrin (0.59 ng/L), followed by bifenthrin (0.98 ng/L), permethrin (< 4.98 ng/L), and chlorpyrifos (12.20 ng/L).

*Comparison of Endpoints for each Species*

Comparing effective concentrations for each species, motility was the most sensitive endpoint across both species. The motility $EC_{50}$ for *C. dilutus* were 1.9 (bifenthrin) to 3.7 (permethrin), and for *H. azteca* 1.1 (permethrin) to 5.5 (cyfluthrin) times lower than corresponding $LC_{50}$. $NOEC_{Motility}$ differed between 1.8 (permethrin) and 2.5 (cyfluthrin) times for *C. dilutus*, and 1.4 (bifenthrin) and 3.9 (permethrin) times for *H. azteca* compared to $NOEC_{Survival}$.

Weight $EC_{50}$ values for *C. dilutus* were 1.2 (cyfluthrin) to 6.7 (bifenthrin) times lower than corresponding $LC_{50}$ values, while the $NOEC_{Weight}$ differed between 2.5 (cyfluthrin) and 3.9 (bifenthrin) times compared to the corresponding $NOEC_{Survival}$. For *H. azteca* weight, the $EC_{50}$ was 1.2 (bifenthrin) to 10.2 (permethrin) times lower than corresponding $LC_{50}$ values, while $NOEC_{Weight}$ differed between 1.4 (bifenthrin) and 3.9 (permethrin) times compared to $NOEC_{Survival}$.

Comparing chemical classes, the type II pyrethroid cyfluthrin represented the most toxic pesticide class, resulting in effective concentrations that were up to 73 times lower than type I pyrethroids [$EC_{50\text{-}Velocity}$ (*H. azteca*) for cyfluthrin compared to permethrin] and 21 times lower than the organophosphate [$EC_{50\text{-}Weight}$ (*H.azteca*) of cyfluthrin compared to chlorpyrifos]. Exposure to

cyfluthrin elicited the greatest effect on motility and growth of both species, and on survival of *C. dilutus*, while bifenthrin was most toxic to *H. azteca* survival.

Compared to *C. dilutus*, *H. azteca* was more sensitive across all pesticides tested. The $LC_{50}$ was up to 50, the $EC_{50}$ for motility up to 43, and $EC_{50}$ for weight up to 12 times lower for *H. azteca* than for *C. dilutus*. The only exception was permethrin for the motility endpoint, where the $EC_{50}$ for *C. dilutus* was 1.2 times lower than for *H. azteca*. The $NOEC_{Motility}$ and $NOEC_{Weight}$ of *H. azteca* were 5 – 43 times and 3 – 11 times lower than for *C. dilutus*, respectively, with bifenthrin displaying the largest and permethrin the smallest difference. While the weight of *H. azteca* was a more sensitive endpoint across all chemicals tested, it displayed the smallest differences in sensitivity between the two species.

## 2.5 Discussion

*Chironomus dilutus* and *H. azteca* differed greatly in their sensitivities to the four pesticides investigated and showed different sublethal responses at fractions of $LC_{50}$ concentrations.

Cyfluthrin was the most toxic pesticide in all endpoints tested for *C. dilutus* and both sublethal endpoints tested for *H. azteca*. Like other type II pyrethroids, cyfluthrin is chemically modified via the addition of functional groups (cyano and halogen group), and therefore hydrolyzes more slowly than type I pyrethroids, resulting in a toxic potency up to 73-times greater than that of the type I pyrethroids investigated in this study. However, *H. azteca* survival was most sensitive to bifenthrin, rather than cyfluthrin, which also caused the biggest difference in species sensitivity in terms of survival ($LC_{50}$ value for *H. azteca* was 50 times lower than for *C. dilutus*) and motility ($EC_{50}$ value for *H. azteca* was 38 times lower than for *C. dilutus*). This difference in sensitivity between the two species was also reported in Weston et al. (2013), where the contribution of pyrethroids to sediment toxicity was investigated. This study found that bifenthrin was approximately twelve-fold more toxic to *H. azteca* than to *C. dilutus* whereas differences among cyfluthrin, permethrin, and chlorpyrifos were only two-fold. Similar results were found in other studies (Maund *et al.* 1998; Amweg *et al.* 2005; Maul *et al.* 2008). Weight was the most sensitive endpoint to detect pyrethroid toxicity using *C. dilutus* in this study. Significant effects on *C. dilutus* weight were observed at concentrations of 9.05 ng/L cyfluthrin, 10.75 ng/L bifenthrin, and 24.77 ng/L permethrin. For *H. azteca*, both sublethal

endpoints were effective to detect low-level pesticide concentrations. The concentrations causing significant effects on all three endpoints in both species are within the range of environmentally relevant concentrations as reported in previous monitoring studies in different states of the USA (Anderson *et al.* 2006; Smith and Lizotte 2007; Werner *et al.* 2010; Phillips *et al.* 2012). For example, studies in Californian creeks by Budd *et al.* (2009) and Weston and Lydy (2012) detected bifenthrin at concentrations up to 37.3 ng/L, permethrin up to 470.0 ng/L, and cyfluthrin up to 8.7 ng/L. Chlorpyrifos was detected at concentrations up to 226.0 ng/L (Weston and Lydy 2010).

*Mortality as an Endpoint*

The determined $LC_{50}$ for the pesticides used in this study match results reported in other studies using *H. azteca*. Brander et al. (2009) reported a 10d $LC_{50}$ for permethrin of 48.90 ng/L (40.90 ng/L in this study), and Deanovic et al. (2013) a 10d $LC_{50}$ for bifenthrin of 2.3 ng/L (2.0 ng/L in this study) and for cyfluthrin 1.9 ng/L (2.89 ng/L in this study), while Phipps et al. (1995) reported a higher 10d $LC_{50}$ for chlorpyrifos of 86.0 ng/L (50.41 ng/L in this study). The difference in chlorpyrifos toxicity is likely caused by a different experimental setup, as Phipps et al. (1995) used a flow-through system, while Deanovic et al. (2013) and Brander et al. (2009) used a static system as was used in this study. No 10d $LC_{50}$ was reported for *C. dilutus* in the literature for cyfluthrin, and values reported for the other three chemicals differed from the ones determined in this study. Ding et al. (2012) determined different $LC_{50}$ of bifenthrin (23.0 ng/L), permethrin (99.0 ng/L), and chlorpyrifos (140.0 ng/L) for *C. dilutus* using a static system, without solution renewal and a decreased feeding interval, which possibly caused the differing values compared to this study.

*Motility as an Endpoint*

Motility was a highly sensitive endpoint to detect toxicity of cyfluthrin and bifenthrin on *H. azteca* below 1 ng/L. Swimming behavior is ecologically important since a reduction could make invertebrates more vulnerable to predation, drift, or food competition (Holomuzki *et al.* 2010). It is an especially relevant endpoint when investigating neurotoxic substances, such as organophosphates and pyrethroids, because paralysis is the first visible symptom of exposure (Rubach *et al.* 2011). Several

studies have demonstrated the suitability of swimming performance for assessing effects of insecticides on fish, as it integrates biochemical and physiological processes, and is an important indicator of fitness in aquatic species (Heath *et al.* 1993; Geist *et al.* 2007; Beggel *et al.* 2010). Motility is not an established endpoint in toxicity testing using invertebrates, but Rubach et al. (2011) who investigated the species sensitivity of 15 arthropod species, including the amphipod *Gammarus pulex*, on exposure to chlorpyrifos, found swimming behavior, rather than mortality, to be the most sensitive endpoint to use for risk assessment of neurotoxic compounds. This was also found in an exposure of the rotifer species *Brachionus calyciflorus* to the organophosphate dimethoate that resulted in adverse effects on the swimming behavior (Chen *et al.* 2014). These results as well as those from our study clearly demonstrate that motility is an important indicator to detect low-level pesticide concentrations which should be considered in ambient water monitoring and regulatory toxicity assessments.

*Weight as an Endpoint*

Pyrethroid exposure resulted in reduced growth of both species. This could have been caused by food avoidance due to pyrethroids bound to organic material or decreased ability to ingest food (Maul *et al.* 2008). Alternatively, feeding rates may have been maintained, in which case reduced growth could be a direct effect of these insecticides; e.g., energetic reserves are allocated toward detoxification (Campero *et al.* 2007). Growth was the most sensitive endpoint for *C. dilutus* in this study, reflecting previously reported results. Maul et al. (2008) investigated the toxicity of bifenthrin, permethrin, and lambda-cyhalothrin on *C. dilutus* and found dramatic growth inhibition within the 10d exposure. Growth is an established endpoint in fish toxicity studies as it represents an important ecological endpoint affecting predator avoidance and reproduction (Haya 1989; Connon *et al.* 2009). For smaller organisms such as invertebrates, growth is likely to be of similar ecological relevance as for fish. For example, reduced larval growth in *C. dilutus* negatively affected pupation, emergence (86 to 100% reduction), adult female size, number of eggs per female, and fecundity (Liber *et al.* 1996; Sibley *et al.* 1997; Ristola *et al.* 1999). Sufficient growth during the larval stages of chironomids, that successfully leads to pupation and emergence, may therefore, be even more crucial than growth of

purely aquatic species such as amphipods (Agra and Soares 2009), as chironomid reproduction occurs during the adult terrestrial stage. Additionally, smaller individuals may also be more susceptible to predators, may have reduced resistance to other environmental stressors as homeostatic energy demands are increased to contend with contaminant stress (Liber *et al.* 1996; Sibley *et al.* 1997; McKenney *et al.* 1998). Therefore, impairment of this endpoint could have profound population-level effects and is thus a highly important endpoint to consider in toxicity testing and ambient water monitoring.

*Differences in Sensitivity of Species*

Chlorpyrifos affected growth of *H. azteca*, but not of *C. dilutus* in this study. Generally, differences in the sensitivity of species to pesticides can be explained by their differences in behavior and habitat, as well as differences in toxicokinetics (uptake, distribution, biotransformation, elimination) and toxicodynamics (interaction with biological target sites) with differences in the mode of action being the most likely explanation in this specific case (McCarty and Mackay 1993; Vaal *et al.* 2000; Rubach *et al.* 2012). The metabolism of pesticides, their target sites, and the binding affinity at target sites, is known to differ even with only slightly different chemical structures (Soderlund *et al.* 2002; Nasuti *et al.* 2003; Vais *et al.* 2003). Variations in toxicokinetics among species can result from differences in lipid content, body size, and respiratory strategy (Baird and Van den Brink 2007; Nyman *et al.* 2014). In addition, the biotransformation capacity of a species to inactivate or activate specifically acting compounds has been considered an important factor causing differences in sensitivity (Chambers and Carr 1995; Escher and Hermens 2002). While both *C. dilutus* and *H. azteca* possess cytochrome P450-mediated mono-oxogenases capable of metabolizing organophosphate insecticides (Ankley and Collyard 1995), metabolic enzyme profiles can vary greatly across species (Clark 1989; Godin *et al.* 2006). As an organophosphate, chlorpyrifos is metabolically activated to a more toxic intermediate, chlorpyrifos-oxon that mainly acts on the nervous system by inhibiting acetylcholinesterase (ACh), leading to continuous neurotransmission, acute cholinergic syndrome and eventually paralysis and death (Hsieh *et al.* 2001). The difference in response to chlorpyrifos exposure

between the two species could result from the capability of *C. dilutus* larvae to withstand an increased inhibition of ACh as shown in previous studies (Rakotondravelo *et al.* 2006; Rebechi *et al.* 2014).

Habitat differences are another major contributing factor to sensitivity differences between chironomids and amphipods. *H. azteca* are epibenthic grazers primarily occurring at the interface of the water column and sediment or detritus (Wang *et al.* 2004), while *C. dilutus* burrow into the sediment and feed on organic particles in the walls of their tube (Proulx and Hare 2014). This could lead to differences in exposure of *C. dilutus* to pyrethroids. Pyrethroids are highly nonpolar chemicals of low water solubility and high $K_{ow}$ values resulting in a high affinity to any type of surface. Laskowski (2002) summarized physical and chemical environmental properties of pyrethroids confirming that Log $K_{ow}$ values for bifenthrin, permethrin, and cyfluthrin are similar, ranging between 6.0 and 6.4. Chlorpyrifos is slightly less hydrophobic than pyrethroids with an log $K_{ow}$ of 4.7 (Kravvariti *et al.* 2010). The binding properties of pyrethroids have been shown to inhibit their degradation (Lee *et al.* 2004), suggesting an accumulation of these compounds in the benthos causing an increased exposure to benthic organisms such as *C. dilutus*. Maund *et al.* (2001), on the other hand, reported that epibenthic and benthic organisms bioaccumulated a similar amount of sediment-bound pyrethroids. This indicates that bioaccumulation may be driven by cuticular uptake of the dissolved fraction, rather than ingestion of or direct contact with pyrethroid-contaminated sediments.

This study supports the use of *C. dilutus* and *H. azteca* as reliable indicators of pyrethroid presence in water samples, however ecological implications cannot be directly assessed from toxicity demonstrated in laboratory species. Different species of chironomids are hard to identify and there are additionally important genetic and physiological differences between laboratory and field populations of both *H. azteca* (Major *et al.* 2013; Weston *et al.* 2013) and chironomids (Hoffman and Fisher 1994; Woodworth *et al.* 2002; Nowak *et al.* 2008). Consequently, the exposure concentrations at which effects were observed in *C. dilutus* and *H. azteca* cannot necessarily be seen as universally valid. In any case, the observed pronounced differences in the sensitivity of both species is not surprising since considerable interspecies variation in response to chemical stress exists for a wide range of animals and plants (Naylor *et al.* 1990; Baird *et al.* 1991; Bridges and Semlitsch 2000; Jensen and Forbes 2001).

## 2.6 Conclusion

Our data highlights the importance and usefulness of integrating sublethal endpoints on invertebrates into water monitoring efforts and ecological risk assessment, especially to evaluate low-level contaminant concentrations. Sublethal endpoints revealed significant effects even below the limit of detection of current-use analytical methods. Our results show that pesticide sensitivities are not easily extrapolated from one species to another, or between chemicals. Environmental risk may therefore be underestimated if surface water bodies are monitored assuming broad representation from a single invertebrate species, from a single test endpoint, or by assuming that similar pesticides have similar effects. Our results demonstrate that the choice of the toxicity test, especially with respect to test species and endpoint, can be crucially important for the detection of insecticide toxicity at low concentrations. It is important to characterize not only the toxicity of common aquatic contaminants, but also the variability in effects across species. Doing so will improve ambient water monitoring efforts and ecological risk assessment by determining the most sensitive species and endpoints that should be used to detect contaminants in water bodies. Understanding the variability in response across species will also help conservation efforts to understand the extent to which species will be affected by contaminant stress.

# 3. The Use of Growth and Behavioral Endpoints to Assess the Effects of Pesticide Mixtures Upon Aquatic Organisms

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## 3.1 Abstract

Aquatic communities are often subject to complex contaminant mixtures, usually at sublethal concentrations, that can cause long-term detrimental effects. Chemicals within mixtures can effectively interact, resulting in synergism, antagonism or additivity. We investigated the tertiary mixture effects of two pyrethroids, lambda-cyhalothrin and permethrin, and the organophosphate chlorpyrifos, evaluating sublethal endpoints; immobility and growth, on *Chironomus dilutus* in 10-day exposures. We utilized a toxic units (TU) approach, based on median lethal concentrations (LC50) for each compound. The concepts of independent action and concentration addition were used to compare predicted mixture toxicity to observed mixture toxicity. Increased immobility resulted from mixture concentrations ≥ 1 TU (7.45 ng/L lambda-cyhalothrin x 24.90 ng/L permethrin x 129.70 ng/L chlorpyrifos), and single pesticides concentrations ≥ 0.25 TU (5.50 ng/L lambda-cyhalothrin, 24.23 ng/L permethrin, 90.92 ng/L chlorpyrifos, respectively). Growth was inhibited by pesticide mixtures ≥ 0.125 TU (1.04 ng/L lambda-cyhalothrin x 3.15 n/L permethrin x 15.47 ng/L chlorpyrifos), and singly by lambda-cyhalothrin ≥ 0.25 TU (5.50 ng/L), and permethrin ≥ 0.167 TU (18.21 ng/L). The no observed effect concentrations (NOEC) for immobility and growth, for both mixture and single-pyrethroid exposure, were up to 8.0 and 12.0 times respectively lower than the corresponding NOEC for survival. The median effective concentrations (EC50) for growth (mixture and single-pyrethroid exposure) were up to 7.0 times lower than the respective LC50. This study reinforces that the integration of sublethal endpoints in monitoring efforts is powerful in discerning toxic effects that would otherwise be missed by solely utilizing traditional toxicity assessments.

## 3.2 Introduction

Aquatic invertebrate communities are generally exposed to multiple stressors that potentially include complex mixtures of contaminants. The impacts of these are of ecological concern, particularly during an organism's sensitive, developmental life stages (Oros and Werner 2005; Geist 2011; Brooks *et al.* 2012; Connon *et al.* 2012). Concentrations of pesticides found in waters that receive agricultural and urban runoff often do not occur at levels that result in direct mortality (Scholz *et al.* 2012). Although these chemicals may be present in aquatic environments at relatively high concentrations during the peak application periods of spring and early summer, water flow and adsorption of pesticides to sediments and surfaces dictate that organisms are rarely exposed to elevated concentrations that cause mortality for continuous periods of time (Phillips *et al.* 2012; Beketov *et al.* 2013; Jeon *et al.* 2013). However, because pesticides and their breakdown products are retained in sediments and may gradually re-dissolve or otherwise remain available at more consistent low levels (e.g., through diet or contact), research on chronic and environmentally typical low-level exposures is needed. Exposure to low-level concentrations for extended periods of time, or to moderate concentrations for multiple brief periods, can potentially result in physiological impairments (Nyman *et al.* 2013; Scherer *et al.* 2013). Examples of reported effects in invertebrate organisms include reduction in emergence (Du *et al.* 2013), case-abandonment (Johnson *et al.* 2008) and reduced growth (Rakotondravelo *et al.* 2006). Many insecticides may have effects that can lead to long-term severe health impacts or reproductive impairment, which are often not detectable using traditional toxicity testing methods (Christensen *et al.* 2005; Connon *et al.* 2012). Effect-based endpoints, designed to assess sublethal impairments are often more sensitive and better predictors of deleterious effects associated with contaminated water and sediments (Maul *et al.* 2008; Connon *et al.* 2012; Deanovic *et al.* 2013; Rasmussen *et al.* 2013).

Even though aquatic organisms are generally exposed to contaminant mixtures, data used in ecotoxicological risk assessment are predominantly based on single substance evaluation (Junghans *et al.* 2006; Backhaus *et al.* 2013; Gregorio *et al.* 2013). This regulatory approach, though informative, may underestimate ecological relevance because the effects of contaminant mixtures is known to

differ from that predicted based on the sum of individual contaminant effects (Backhaus *et al.* 2000; Altenburger *et al.* 2004; Nørgaard and Cedergreen 2010). Two fundamental concepts exist, which are devised to evaluate the general relationships between the effects of single substances and their corresponding mixtures: concentration addition (CA), in which the effect of each contaminant can be expressed as if it were a dilution of the other and is based on the assumption of a similar action, and independent action (IA), which is based on the assumption of probabilistic independence of the effects of dissimilarly acting agents (Faust *et al.* 2000; Backhaus *et al.* 2004; Belden *et al.* 2007; Cedergreen 2014). In brief, if chemical effects do not interact in a mixture, the influences of each compound are effectively additive and the actual effect of the mixture is adequately described by either CA or IA as a reference model. However, interactions may occur and chemicals in a mixture may produce effects that are synergistic (more severe) or antagonistic (less severe), than predicted by either reference model (Lydy *et al.* 2004; de Zwart and Posthuma 2005; Jonker *et al.* 2005; Belden *et al.* 2007). Additive, synergistic and antagonistic responses have been documented for mixtures of various classes of pesticides, and the majority of studies that have been conducted, have focused on binary mixtures. Our study expands this approach to include tertiary mixtures of contaminants that are often detected in surface waters worldwide, and makes use of sublethal endpoints that are likely to have substantial ecological relevance.

We investigated the combined effects of three commonly used insecticides: two pyrethroids; permethrin (type I pyrethroid) and lambda-cyhalothrin (type II pyrethroid), and one organophosphate; chlorpyrifos, on survival and the sublethal endpoints of immobility and growth of *Chironomus dilutus* following a 10-day exposure. *C. dilutus* is used as a standard invertebrate species used in toxicity testing, and is among the numerous non-target species that are potentially affected by pesticide runoff. It has been shown to be highly sensitive to pyrethroids and organophosphates in field and laboratory studies (Weston *et al.* 2004; Anderson *et al.* 2006; Du *et al.* 2013; Li *et al.* 2013). The life cycle of *C. dilutus* is comprised of three aquatic stages (egg, four larval instars and pupae) and a terrestrial adult stage. The larval stage is representative of organisms living in the benthic zone. During this stage organisms burrow in the upper sediments, and utilize organic matter and sediment particles to build

their protective cases. Like many benthic organisms, they feed on detrital particles, making them ideal organisms for testing the sediment-water interphase.

We selected the three insecticides because they are among the most frequently detected insecticides in aquatic habitats worldwide (Amweg *et al.* 2006; Sprague and Nowell 2008; Hintzen *et al.* 2009; Trimble *et al.* 2009; Bereswill *et al.* 2013; Li *et al.* 2013), and are known to be highly toxic to aquatic invertebrates and fish species (Werner *et al.* 2010; Phillips *et al.* 2012). All three pesticides are used for similar pest treatments such as the cultivation of vegetables, fruits, grains, and for landscape maintenance, and were repeatedly detected in the same water or sediment samples in recent studies (Weston *et al.* 2008; Budd *et al.* 2009; Weston *et al.* 2013). The selected pesticides are all neurotoxins with different neurological target sites and/or modes of action. Although modulations of the voltage-gated sodium channels are similar between the two types of pyrethroids, the degree of modification of sodium currents is different. Single sodium channel currents are prolonged to a greater extent with type II than type I pyrethroids (Wouters and van den Bercken 1978; Clark and Matsumura 1982; Nasuti *et al.* 2003). Organophosphates (chlorpyrifos) on the other hand, inhibit acetylcholine esterase activity (Malison *et al.* 2010; Hua *et al.* 2013) directly impacting the synaptic signal. Varying modes of action could drive potential interactive exposure effect differences between single chemicals and complex mixtures.

Given that pesticides predominantly occur at concentrations below those that cause mortality, sublethal endpoints are more applicable at evaluating environmental relevance. By assessing the effects of the three pesticides in 10-day toxicity tests, we evaluate the use and effectiveness of sublethal endpoints in mixture toxicity testing for regulatory applications and monitoring studies.

## 3.3 Materials and Methods

*Test Organisms and Acclimation*

*Chironomus dilutus* were obtained from Aquatic Biosystems, Fort Collins, CO, USA. Under typical laboratory conditions, *C. dilutus* begin to pupate, and emerge as adults 21 days after hatching, thus 2$^{nd}$ instar larvae (8-10 days old) were used as to avoid emergence during the 10-day exposures.

Upon arrival, dissolved oxygen (DO > 2.5 mg/L), and temperature (T° 23 ± 2 ºC) of transport water sub-samples were measured, and were within acceptable ranges stipulated by U.S. EPA standard test protocols (U.S.EPA 2000). Healthy animals were moved to aerated 7-L aquaria, fed, and acclimated to laboratory test conditions for 48h. During the acclimation period, approximately 50% of the transport water was changed twice daily and refilled with test control water, i.e., deionized water, modified to attain U.S. Environmental Protection Agency (U.S. EPA) moderately hard specifications (hardness 90-100 mg/L $CaCO_3$, alkalinity 50-70 mg/L as $CaCO_3$, SC 330-360 µS/cm and pH 7.8-8.2) (Eide and Johansson 1994; U.S.EPA 2000, 2002). *C. dilutus* were fed 10 ml of 4 g/L Tetramin® slurry daily.

*Chemicals and Chemical Analysis*

Chlorpyrifos (purity > 98%, CAS number 2921-88-2), lambda-cyhalothrin (purity > 98%, CAS number 91465-08-6), and permethrin (purity > 98%, CAS number 52645-53-1) were purchased from Chem Service (West Chester, PA, USA). Pesticide stock solutions were prepared in methanol and spiked into laboratory control water to achieve exposure concentrations illustrated in Table 4 and Table 5 with a final methanol concentration of 0.05%.

Before adding the pesticide solutions into the test beakers, three 1-L water samples for each single-chemical exposure and the tertiary mixture exposure were collected, and stored at 4°C for subsequent chemical analyses. Within 24 h, the samples were extracted by solid phase extraction (Supelclean™ ENVI™ - 18 SPE Tubes, 500 mg, Sigma-Aldrich, St. Louis, MO, USA), and analyzed using an HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an HP-5973N quadrupole mass spectrometer detector, operated in electron capture negative ionization mode (GC-ECNI-MS) with methane as the reagent gas, equipped with a split-splitless injector (280°C, splitless, 1.5-minute purge time) and a Supelco DB-5MS column (30 m x 0.25 mm with a 0.25 µm film thickness). Instrumental calibration was performed using nine sets of calibration standard solutions containing all three pesticides (each purchased as 100 µg/ml solution in acetonitrile, Chem Service, West Chester, PA), the surrogate trans-permethrin D6 (EQ Laboratories, Atlanta, GA), and an internal standard; 4-4’ dibromo-octafluorobiphenyl (Chem Service, West Chester, PA) in hexane.

Quantity was calculated based on peak area and comparing them to the standard curves. Quality-assurance/quality-control was conducted by analyzing a method blank of deionized water (Milli-Q) to ensure that no contamination occurred during sampling extraction and analysis. The surrogate trans-permethrin D6 was added to each sample, including the blank, to monitor matrix effects and overall method performance. Surrogate recoveries were on average 103% with a range between 79 – 112% indicating high extraction efficiency. Reported values were not corrected for surrogate recovery. 4-4' dibromo-octafluorobiphenyl was added to sample extracts before analysis in order to correct quantitative differences in extract volume as well as to monitor instrument conditions. No pesticides were detected in the control or the method blank. Measured concentrations by GC-ECNI-MS were lower than nominal concentrations; average recoveries for lambda-cyhalothrin were 65% (range 62 - 86%), for permethrin 46% (range 34 - 58%), and for chlorpyrifos 78% (range 71 - 86%). The average proportion of the measured concentrations recovered for each pesticide was used as a factor to estimate the realized concentrations for each exposure (Tables 1 and 2), and are presented as measured concentrations hereon.

*Exposure Tests*

Mixture toxicology employs a dimensionless ratio, the toxic unit (TU), to generate a normalized scale (McCarty *et al.* 1992). Each toxicant concentration is considered as a fraction of its individual toxicity, which most commonly is expressed in terms of its LC50. The total TU of a mixture is the sum of the individual fractions. The TU approach assumes dose additivity (Altenburger *et al.* 2004). Thus, the sum of TU describes the joint chemical concentration of a mixture, given a known common effect concentration, which can be used to assess the toxicity of a tertiary mixture as follows:

$$TU_{summation} = \frac{Cw_1}{LC50_1} + \frac{Cw_2}{LC50_2} + \frac{Cw_3}{LC50_3} \quad (1)$$

where $Cw_i$ is the concentration of chemical 1 in a mixture and $LC50_i$ is the $LC50_{96h}$ for chemical 1 (McCarty *et al.* 1992). For example, the sum of 1/3 of the $LC50_{96h}$ of each pesticide equals a TU value of 1. Pesticides were combined in an attempt to produce equitoxic mixtures and to evaluate their interactive effects (Belden and Lydy 2006; Symington *et al.* 2011). The treatments used in the toxicity

tests were based on expected $LC50_{96h}$ values determined by other research groups using similar test methods. Targeted nominal $LC50_{96h}$ values for *C. dilutus* were 189.00 ng/L permethrin (Harwood *et al.* 2009), 37.90 ng/L lambda-cyhalothrin, and 470.00 ng/L chlorpyrifos (Ankley and Collyard 1995). Based on each reported $LC50_{96h}$ value, we exposed animals to single-pesticide TU values of 0.167, 0.25, 0.33, 0.5, 1, 1.5, and 3 (Table 1), and the following mixture TU values: 0.125, 0.25, 0.5, 0.75, 1, 1.5, and 3 (Table 2). These concentrations were specifically chosen to include environmentally typical concentrations as reported in previous studies, as well as high values that may occur transiently (Anderson *et al.* 2006; Budd *et al.* 2009; Werner *et al.* 2010; Weston and Lydy 2010). Single-pesticide responses were then used to predict the combined mixture toxicity, and compared to the observed mixture response (see Statistical Analysis).

Mixture and single-chemical exposures were conducted at the same time over a 10-day period using the same batch of animals ($2^{nd}$ instar larvae). Tests were conducted at 23 ± 2 ºC with a 16-h light: 8-h dark photoperiod, and consisted of four replicate 1 L glass beakers, each containing a layer of 10 g clean and autoclaved silica sand as a substrate, 750 ml of treatment water, and 10 organisms. The sand allowed chironomids to build their cases. Once test solutions were added to the test vessels at test initiation, organisms were randomly placed into each beaker.

Mortality was recorded daily, at which time 80% of each test solution was renewed, and any dead organisms and debris were removed from the test vessels. Water quality parameters (pH, specific conductance, DO, and T°) of renewal water and wastewater were measured. At test initiation and after each water renewal, organisms were fed 1.5 ml of 4 g/L Tetramin® slurry. Test vessels were randomly redistributed within the exposure chamber at each renewal day.

Mobility of each organism was determined at test termination (day 10) using video analysis. Chironomids are generally sedentary if food and oxygen are sufficient in the immediate area, but are inclined to be mobile when they are not provided substrate. Therefore, surviving organisms were carefully teased from their cases and transferred individually into corresponding filming chambers; a 5.1 cm diameter well in a five-welled white PVC plate containing corresponding treatment water without substrate. To improve lighting quality and contrast of the videos, the white PVC plate was then placed on a light board. The positioning of the plate and video settings were standardized for

each recording. Videos were recorded in MPEG-2 format, using a Panasonic® black and white CCTV Camera (12V DC) filming all five filming chambers from the top. The camera was connected to a portable laptop-computer via a USB Frame grabber (Model WinTV-HVR 950, Hauppauge Computer Works, Hauppauge, NY). Thirty frames per second were collected for each organism, over an 80 second period. Recorded videos were then analyzed using Ethovision XT 6.1 Software (Noldus Information Technology Inc., Leesburg, VA) to determine percentage immobility.

Weight was determined using the same organisms used for mobility assessments. Following video recording, the organisms were transferred from the filming chambers onto pre-weighed tin dishes (pooled per treatment replicate), desiccated at 60°C following methods described by Nahon *et al.* (2010), and weighed using a Mettler® AE 100 balance (0.1 mg accuracy). To examine 10-day growth, the weights of a separate set of four replicates of ten organisms were measured at test initiation and compared to the weights of surviving individuals at test termination. Weight is presented as mg/surviving individual. Data for both sublethal endpoints is presented up to 1 TU as mortality in higher treatments was greater than 50%.

*Statistical Analysis*

Median lethal and effective concentrations (LC50/EC50) and no observed effect concentrations (NOEC) were determined for mortality and the sublethal endpoints of immobility and growth, based on measured exposure concentrations (Table 4 and Table 5).

Table 4 Measured and nominal concentrations of lambda-cyhalothrin, permethrin, and chlorpyrifos at test initiation for single-chemical exposures of *C. dilutus* over 10 days. * indicates that concentrations were calculated based on the average proportions recovered in analytical tests for each exposure. Average factors used for each chemical: 0.58 for lambda-cyhalothrin, 0.51 for permethrin, and 0.77 for chlorpyrifos. 'TU' for each pesticide represents the toxic unit for the lethal concentration that was chosen for each treatment for each single pesticide

| | Pesticide Concentration (ng/L) | | | | | |
|---|---|---|---|---|---|---|
| TU of single pesticide | Lambda-Cyhalothrin | | Permethrin | | Chlorpyrifos | |
| | Nominal | Measured | Nominal | Measured | Nominal | Measured |
| 0.167 | 6.32 | 4.39 | 31.50 | 18.21 | 78.33 | 55.89 |
| 0.25 | 9.48 | 5.50* | 47.25 | 24.10* | 117.50 | 90.48* |
| 0.33 | 12.63 | 7.10 | 63.00 | 30.45 | 156.67 | 117.84 |
| 0.50 | 18.95 | 9.34 | 94.50 | 45.08 | 235.00 | 201.07 |
| 1.00 | 37.90 | 21.98* | 189.00 | 96.39* | 470.00 | 361.90* |
| 1.50 | 56.85 | 32.97* | 283.50 | 144.59* | 705.00 | 542.85* |
| 3.00 | 113.70 | 65.95* | 567.00 | 289.17* | 1410.00 | 1085.70* |

Table 5 Measured and nominal concentrations of lambda-cyhalothrin, permethrin, and chlorpyrifos in the mixture exposures at test initiation for mixture exposures of *C. dilutus* over 10 days. * indicates that concentrations were calculated based on the average proportions recovered in analytical tests for each exposure. Average factors used for each chemical: 0.66 for lambda-cyhalothrin, 0.40 for permethrin, and 0.79 for chlorpyrifos. 'TU for each pesticide equals three times 'TU of mixture' and represents the percentage lethal concentration that was chosen for each treatment for each pesticide

| | | Pesticide Concentration (ng/L) | | | | | |
|---|---|---|---|---|---|---|---|
| TU of mixture | TU of each pesticide | Lambda-Cyhalothrin | | Permethrin | | Chlorpyrifos | |
| | | Nominal | Measured | Nominal | Measured | Nominal | Measured |
| 0.125 | 0.042 | 1.58 | 1.04* | 7.88 | 3.15* | 19.58 | 15.47* |
| 0.25 | 0.083 | 3.16 | 2.09* | 15.75 | 6.30* | 39.17 | 30.94* |
| 0.50 | 0.167 | 6.32 | 5.44 | 31.50 | 14.50 | 78.33 | 55.32 |
| 0.75 | 0.25 | 9.48 | 6.26* | 47.25 | 18.90* | 117.50 | 92.83* |
| 1.00 | 0.33 | 12.63 | 7.45 | 63.00 | 24.90 | 156.67 | 129.70 |
| 1.50 | 0.50 | 18.95 | 10.25 | 94.50 | 31.94 | 235.00 | 193.40 |
| 3.00 | 1.00 | 37.90 | 25.01* | 189.00 | 75.60* | 470.00 | 371.30* |

We tested for significant differences of the treatments compared to the controls using Analysis of Variance, or where parametric assumptions were not met, a Kruskal Wallis test, including a Dunnett's post hoc comparison, using Minitab 16 Statistical Software 2010 (Minitab, Inc., State College, PA, USA). The significance level or $\alpha$ used in all these tests was $P \leq 0.05$. All differences discussed below

are significant unless otherwise noted. Concentrations that caused a 50% reduction in survival (LC50) and growth (EC50) were determined by fitting non-linear regression curves to the toxicity data using the DRC package version 2.3-96 (Ritz and Streibig 2005) R (R Core Team 2013).

EC50 concentrations for the effects on immobility and for chlorpyrifos on weight were not calculated because the levels of responses did not amount to 50% relative to the control. For all data, log-normal, log–logistic and Weibull functions were fitted with the optimal model fit chosen for each dataset by the distribution that had the lowest Akaike's Information Criterion value. The fit of the optimal model was confirmed by a goodness of fit test. Three-parameter regression models were fitted, assuming a lower limit of 0.

The dose-response data were described with a log-logistic dose-response model with an upper limit of 1:

$$y = \frac{1}{1+ (x/e)^b} \quad (2)$$

Where *e* is the effect concentration (LC50), and the parameter *b* denotes the relative slope around *e*. Once the dose-response curves were fitted, joint effect predictions in relation to concentration addition (CA) and independent action (IA) were made. For the prediction of mixture toxicities the complete concentration-response range between 1 and 99% effect was predicted according to both the CA as well as the IA concept. CA assumes that the chemicals act as dilutions of each other (Bliss 1939; Hewlett and Plackett 1952). For the concept of CA the calculation was conducted as outlined in Backhaus *et al.* (2000). Briefly, the total concentrations of each mixture were calculated in steps of 1% as:

$$LCx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i}\right)^{-1} \quad (3)$$

Where $p_i$ is the fraction of compound *i* present in the mixture. The resulting 99 pairs were connected with straight lines, visualizing the predicted concentration response-curve.

To calculate the mixture effects according to IA, the individual compounds were expressed as fractions *pi* of the total concentration *c*mix. The overall effect of any given total mixture concentration can be calculated as

$$L(c_{mix}) = 1 - \prod_{i=1}^{n}[1 - F_i(c_i)] \quad (4)$$

where $L(c_{mix})$ denotes the predicted effect (scaled from 0-1) of an *n*-compound mixture, $c_i$ is the concentration of the *i*th compound, and $F_i$ is the effect of that concentration if the compound is applied singly. To calculate the mixture effects predicted according to IA, the 99 predicted mixture concentrations obtained from the concentration-response range for the CA model described in Equation (3) were used.

The observed data were considered to be significantly different from the predicted model if the 95% confidence intervals of the observed toxicity values did not overlap the value predicted by the model.

The toxicity of each of the chemicals in a mixture can differ substantially, therefore each chemical's relative contribution to toxicity was expressed by using toxicity units rather than their actual concentrations (LC50) to predict the joint toxicity (Lydy and Austin 2004; Jonker *et al.* 2005). An LC50 resulting in a TU greater than 1 represents a synergistic type of joint action, whereas a TU less than 1 indicates an antagonistic type of action (Pape-Lindstrom and Lydy 1997; Sørensen *et al.* 2007).

## 3.4 Results

Water quality parameters remained stable throughout all exposures and mean control survival of *C. dilutus* was 75% (SE = ± 0.13) meeting the U.S. Environmental Protection Agency minimum acceptance criteria for this species (U.S.EPA 2000). Control mortality was only observed within 24h after test initiation, and was likely caused by random handling stress when transferring the chironomids into treatment beakers.

*Effects on Survival*

The tertiary mixture exposure resulted in a less severe response than the pesticides applied singly. In single-chemical tests, lambda-cyhalothrin was the most toxic pesticide to *C. dilutus* resulting in an LC50 value of 32.99 ng/L (± 2.56 SE), followed by permethrin (159.41 ng/L, ± 16.36 SE) and chlorpyrifos (571.49 ng/L, ± 88.68 SE) (Table 6). These values were 1.1 – 1.5 times higher than the corresponding single-pesticide NOEC values for lambda-cyhalothrin, permethrin, and chlorpyrifos (21.98 ng/L, 144.59 ng/L, and 361.90 ng/L, respectively). The LC50 value for the observed mixture exposure was 1.90 TU (± 0.28 SE), indicating an antagonistic response (greater than 1 TU).

Table 6 Effect concentrations calculated for 10-day exposures of *C. dilutus* to lambda-cyhalothrin (LC), permethrin (Perm), and chlorpyrifos (CLF) applied singly and in mixture (Mixture) of *C. dilutus.* NOEC = no observed effective concentration, LC50 = lethal concentration resulting in 50% mortality of the population, EC50 = effect concentration resulting in 50% reduction in growth, SE = standard error. '---' indicates value was not calculable

| Chemical | Effect concentration (ng/L) | | | | | | |
|---|---|---|---|---|---|---|---|
| | $NOEC_{Survival}$ | LC50 | SE | $NOEC_{Weight}$ | $EC50_{weight}$ | SE | $NOEC_{Immobility}$ |
| LC | 21.98 | 32.99 | 2.56 | 4.39 | 18.13 | 5.20 | < 4.39 |
| Perm | 144.59 | 159.41 | 16.36 | < 18.21 | 22.51 | 7.75 | < 18.21 |
| CLF | 361.90 | 571.49 | 88.68 | --- | --- | --- | < 55.89 |
| Mixture | 1.50 TU | 1.90 TU | 0.28 | 0.125 TU | 0.49 TU | 0.19 | 0.50 TU |

Survival of *C. dilutus* was reduced in concentrations ≥ 1.5 TU of lambda-cyhalothrin (32.97 ng/L) and chlorpyrifos (542.85 ng/L), whereas the mixture exposure caused no significant reduction in survival ≤ 1.5 TU (10.25 ng/L lambda-cyhalothrin, 31.94 ng/L permethrin, 193.40 ng/L chlorpyrifos) indicating an IA response (Figure 7).

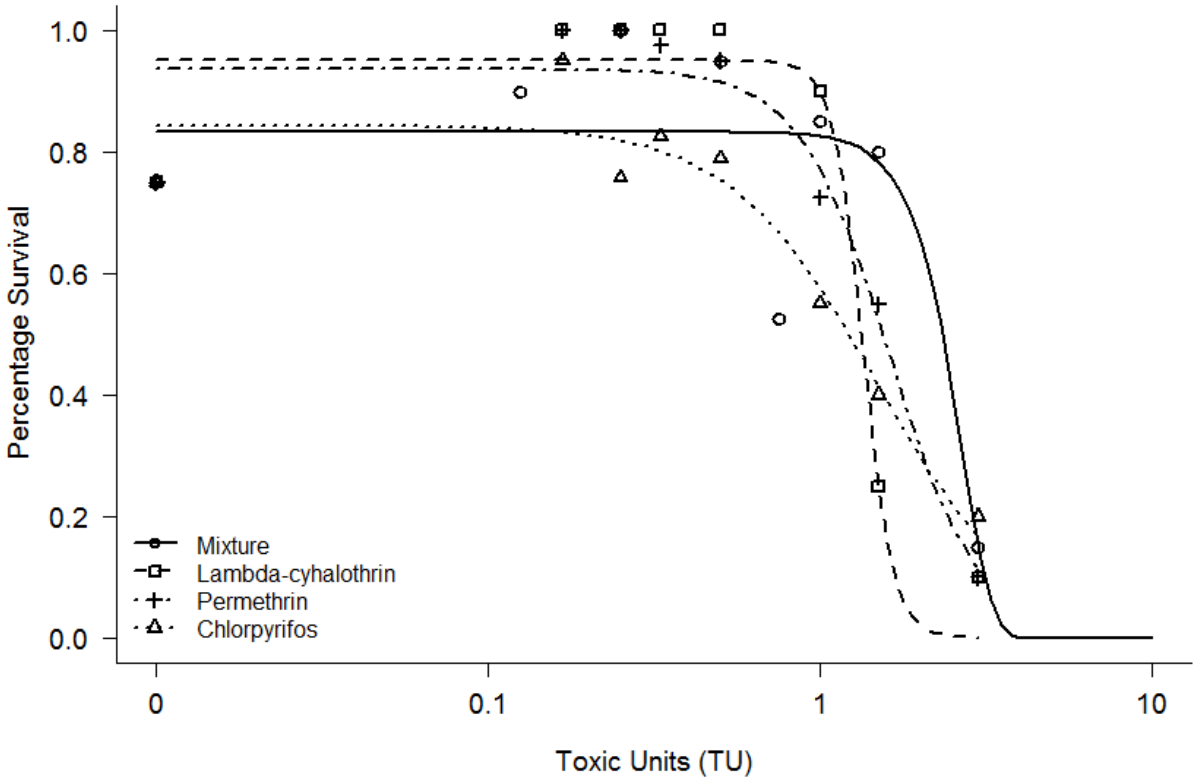


Figure 7 Percent survival of *C. dilutus* following 10-day exposures to lambda-cyhalothrin, permethrin and chlorpyrifos, and corresponding mixtures up to 3 TU. Vertical bars indicate standard errors, * indicates $p < 0.05$ (Dunnett's test). Non-linear regression curves were fitted to the toxicity data using the DRC package version 2.3-96 (Ritz and Streibig 2005) R (R Core Team 2013). Average control survival = 75%

The CA and IA concept were applied to predict mixture toxicity based on the single-pesticide exposures, and compared to the observed mixture response (Figure 8). The observed mixture response was most consistent with the IA concept, whereas the CA concept overestimated the combined effect.

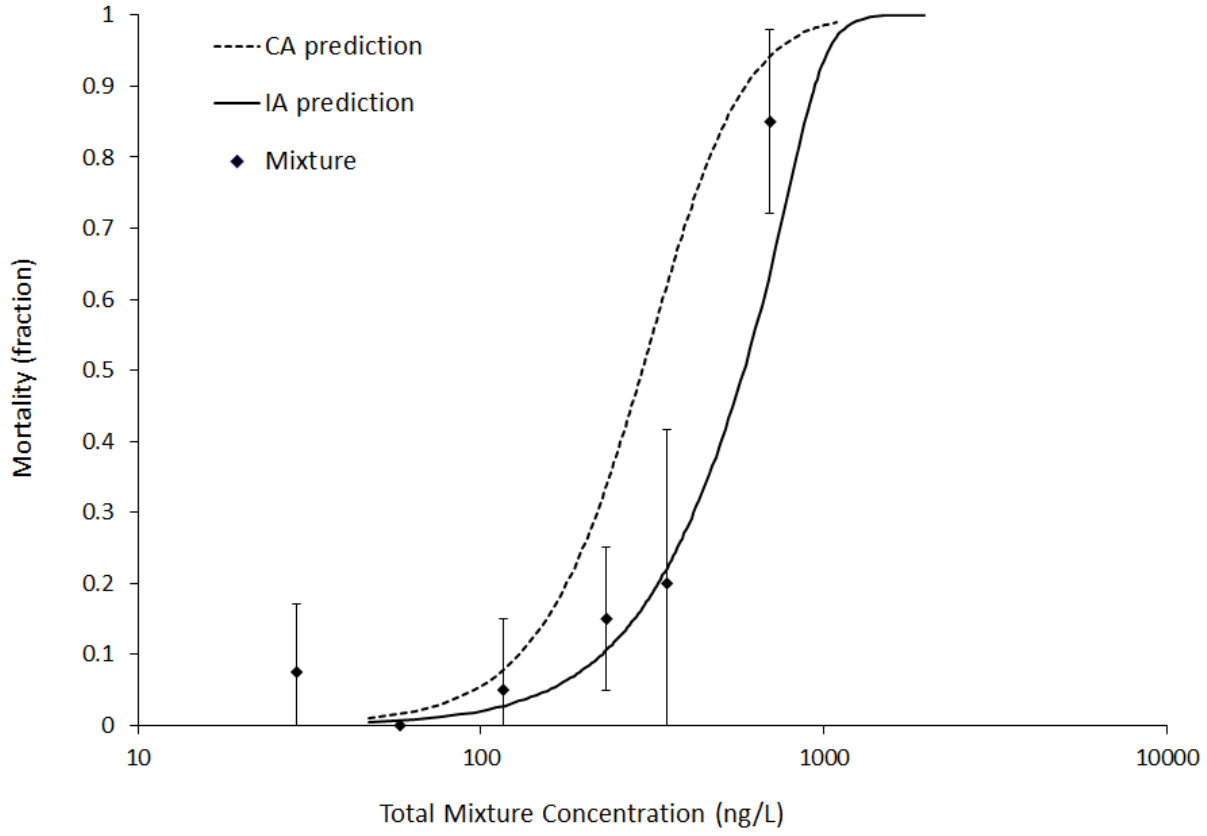


Figure 8 Observed and predicted toxicities of tertiary mixtures of lambda-cyhalothrin, permethrin and chlorpyrifos on *C. dilutus*. Mixture concentrations derived from LC50 values of the individual components. Mixture = observed toxicity of the tertiary mixture; CA prediction = Prediction according to concentration addition model; IA prediction = Prediction according to independent action model

*Effects on Mobility*

Average control immobility was 77.89% (± 6.69 SE) over the recording time of 80s. Single-pesticide exposure caused a decrease in mobility across all concentrations tested with an average immobility of 93.68% (± 0.44 SE) at 0.25 TU for all three pesticides (Figure 9). The exposure to the tertiary mixtures decreased mobility at 1 TU with an average immobility of 94.23%. Exposure to chlorpyrifos individually caused the greatest inhibition of mobility at 1 TU (361.90 ng/L), with an average immobility of 93.15% (± 0.71 SE), followed by permethrin (90.42%, ± 0.59 SE), mixture (88.58%, ± 0.99 SE), and lambda-cyhalothrin (87.48%, ± 1.79 SE). Comparing NOEC values for immobility and survival, permethrin exposures caused the greatest difference (NOEC for immobility 8 times smaller than for survival), followed by chlorpyrifos (6.5 times), lambda-cyhalothrin (5 times), and the mixtures (3 times).

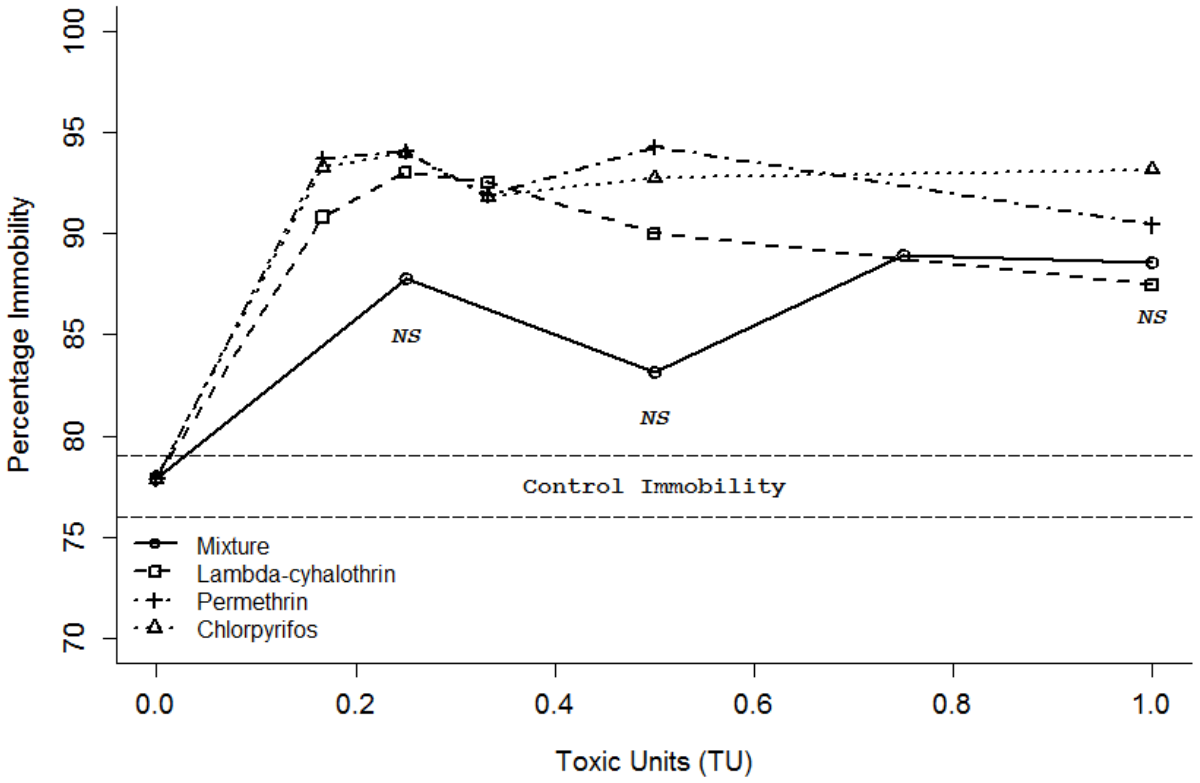


Figure 9 Percentage immobility over 80 s of recording time of *C. dilutus* following 10-day exposures to lambda-cyhalothrin, permethrin, chlorpyrifos, and corresponding mixtures up to 1 TU (survival ≥ 50%). NS = non-significant to controls, all others $p < 0.05$. Data points are connected to aid visualization. Average control Immobility = 77.88%

*Effects on Weight*

Initial weight ($T_0$) of four subsamples of *C. dilutus* was 0.345 mg/individual (± 0.04 SE; Figure 10). Final weight of control organisms was 1.932 mg/individual (± 0.07 SE) equaling a 5.6-fold growth over the 10-day test period. Both pyrethroids and the mixture led to an inhibition in *C. dilutus* growth, while chlorpyrifos had no detectable effect on this endpoint. Compared to the final control weight, reductions were recorded at concentrations ≥ 0.25 TU for lambda-cyhalothrin (from 32.6% up to 65.8% in the highest concentration) and the mixture (25.2 – 63.1%), and ≥ 0.167 TU for permethrin (33.0 – 77.8%).

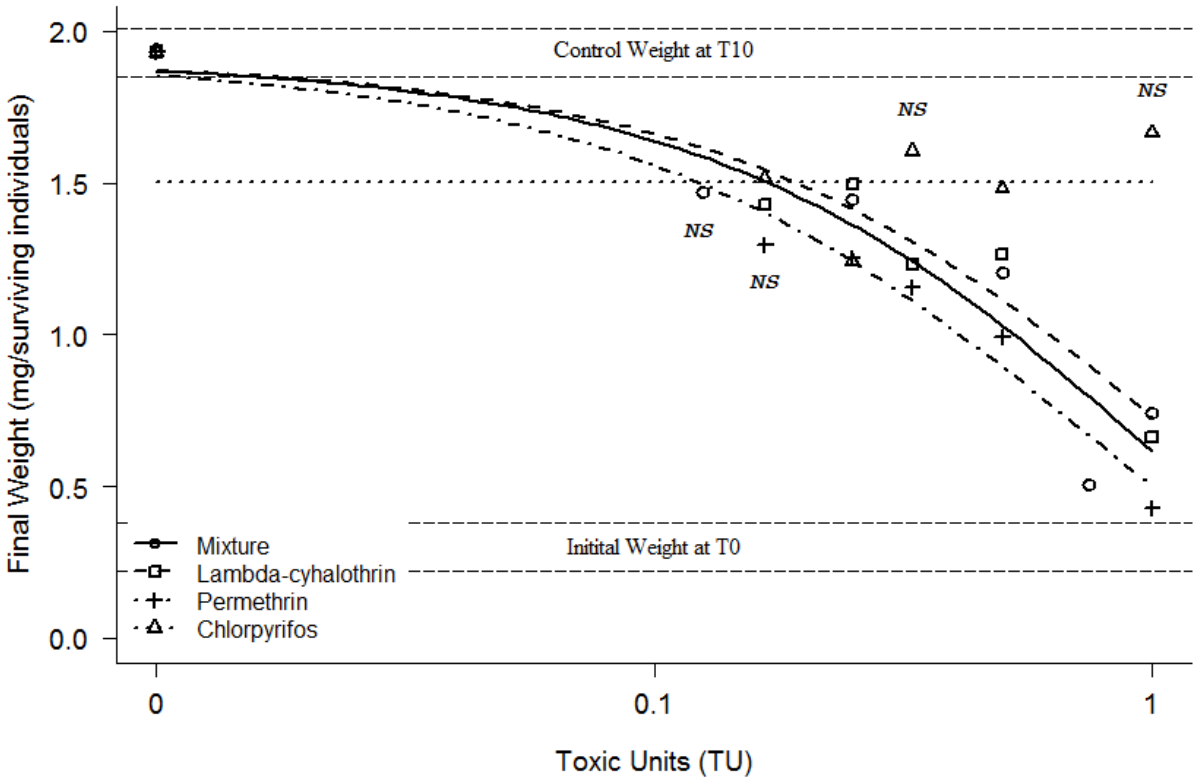


Figure 10 Final weight in mg/surviving individual of *C. dilutus* following 10-day exposures to lambda-cyhalothrin, permethrin, chlorpyrifos, and corresponding mixtures up to 1 TU (survival ≥ 50%). NS = non-significant to controls, all others $p < 0.05$. Non-linear regression curves were fitted to the toxicity data using the DRC package version 2.3-96 (Ritz and Streibig 2005) R (R Core Team 2013). Horizontal bars represent average initial weight at test initiation (T0) (0.345 mg/individual) and average weight of the control at test termination (T10) (1.932 mg/surviving individual), and are presented to facilitate the comparison with initial and final weights along increasing toxic units

In detail, exposure to lambda-cyhalothrin resulted in a growth inhibition up to a final weight of 0.661 mg/surviving individual (± 0.08 SE) at 1 TU (21.98 ng/L lambda-cyhalothrin). Mixture exposures resulted in a final weight of 0.712 mg/surviving individual (± 0.03 SE) at 1 TU (7.45 ng/L lambda-cyhalothrin, 24.90 ng/L permethrin, 129.70 ng/L chlorpyrifos). Exposure to permethrin resulted in a final weight of 0.429 mg/surviving individual (± 0.03 SE) at 1 TU (96.39 ng/L permethrin). NOEC values for the growth endpoint, for both mixture and single-pyrethroid concentrations were 5.0 to 12.0 times lower than the respective NOEC for survival (Table 3), with the mixture exposure (NOEC = 0.125 TU) causing the greatest difference (12 times lower than for survival), and permethrin (NOEC < 18.21 ng/L, 8 times lower) and lambda-cyhalothrin (NOEC = 4.39 ng/L, 5 times lower) the smallest differences. EC50 values for growth were on average 1.8 to 7.0

times lower than the respective LC50 for survival, with permethrin (EC50 = 22.51 ng/L, ± 7.75 SE) representing the largest difference and lambda-cyhalothrin (EC50 = 18.13 ng/L, ± 5.20 SE) the smallest. The EC50 value for the mixture (0.49 TU) was 3.9 times lower than the respective LC50 (1.90 TU).

### 3.5 Discussion

The assessment of three insecticides, lambda-cyhalothrin, permethrin, and chlorpyrifos, demonstrates that mixtures can affect survival in an antagonistic manner (1.90 TU) and result in effects on sublethal endpoints that are up to 12.0 times lower than the corresponding NOEC for survival. The sublethal effect concentrations for growth inhibition caused by lambda-cyhalothrin (EC50 = 18.13 ng/L), permethrin (EC50 = 22.51 ng/L) and the tertiary mixture (0.25 TU = 2.09 ng/L lambda-cyhalothrin, 6.30 ng/L permethrin, and 30.94 ng/L chlorpyrifos) in this study are within the range of environmentally relevant concentrations reported in previous monitoring studies in different states of the USA (Anderson *et al.* 2006; Smith and Lizotte 2007; Werner *et al.* 2010). For example, studies in Californian streams by Budd *et al.* (2009) and Weston *et al.* (2014) detected lambda-cyhalothrin at concentrations of 1.4 to 27.0 ng/L, and permethrin between 4 to 470 ng/L. Chlorpyrifos was detected at concentrations between 1.2 to 226.0 ng/L (Weston and Lydy 2010). Even though concentrations for lambda-cyhalothrin in these monitoring studies were on average lower than for the other two pesticides, the lower effective concentrations of 18.13 ng/L (EC50 Growth) and < 3.69 ng/L (NOEC Immobility) determined in this study suggest that lambda-cyhalothrin is the most toxic, and of the three, the pesticide of greatest concern in terms of potential ecotoxicological effects on invertebrate populations and aquatic communities.

The EC50 of the mixture of 0.49 TU indicates that 0.163 TU (LC8) of each chemical in a tertiary mixture results in a 50% growth inhibition relative to controls. The levels of 0.167 TU as measured in our single-chemical exposures were 3.67 ng/L lambda-cyhalothrin, 16.07 ng/L permethrin, and 60.31 ng/L chlorpyrifos; these too are lower than the reported environmentally relevant concentrations. These results highlight the pressing need to adequately assess the sublethal effects of contaminant mixtures as they co-occur in the environment.

Treatment concentrations for each pesticide were initially chosen from literature values to achieve equitoxic concentrations in each mixture, the measured concentrations were, as anticipated, below nominal concentrations, and toxicity levels are known to vary among studies (Wheelock *et al.* 2005). Differences between nominal and measured concentrations are not unusual, especially when target concentrations are near the limit of detection (Farmer *et al.* 1995; Amweg *et al.* 2005). Wheelock *et al.* (2005) showed that up to 50% of the pyrethroid can adsorb to the sampling container in 24h which may be one explanation for the lower measured concentrations in our study.

The realized toxicity levels were therefore not strictly equitoxic. An equitoxic concentration approach was originally chosen to evaluate the joint action of the tertiary mixture by using CA and IA models, and to determine the degree of interaction, similarly to other studies (Denton *et al.* 2003; Belden and Lydy 2006; Symington *et al.* 2011; Larras *et al.* 2013; Norwood *et al.* 2013; Villa *et al.* 2014). The CA and IA concepts are usually evaluated using a "fixed ratio design", where the constituents ratio is kept constant throughout the studies (Barata *et al.* 2006), however, strictly equitoxic ratios are not essential for mixture toxicity assessments, and non-equitoxic ratios are more reflective of pesticide levels in the environment (Altenburger *et al.* 2004; Brodeur *et al.* 2014).

Ecotoxicological effects are known to differ significantly depending on routes of uptake, e.g. aqueous versus dietary exposures, as demonstrated in Werner *et al.* (2002). *C. dilutus* are detritivores, therefore dietary exposure to contaminants is likely (Laskowski 2002; Liu *et al.* 2004; Yang *et al.* 2006). Pyrethroids and organophosphates are known to adsorb to particulate matter. *C. dilutus* spends most of its larval stage in the substrate, therefore aqueous exposure may be reduced for this species, but cuticular exposure from the substrate may be greater. Pyrethroid exposure resulted in reduced growth of *C. dilutus,* which could have been caused by food avoidance due to insecticide-bound organic material. Alternatively, feeding rates may have been maintained, in which case reduced growth could be a direct effect of these insecticides; e.g., energetic reserves are allocated toward detoxification. Sublethal, behavioral effects such as case abandonment in the caddisfly *Brachycentrus americanus*, has been reported on exposures to esfenvalerate (type II pyrethroid), and associated with energetically costly activities such as case-rebuilding (Johnson *et al.* 2008). Induced case-rebuilding has further been associated with reduced growth in the caddisfly *Odontocerum albicorne* (Stevens *et*

*al.* 1999). Growth- and/or behaviorally-related effect concentrations will likely impact population dynamics.

This could occur through several mechanisms, e.g., Chironomids are short-lived as adults, and since fecundity is largely determined by size at emergence, contaminant exposure may lead to reduced number of offspring in subsequent generations (Xue and Ali 1994). Smaller individuals may also be more susceptible to predators, may have reduced resistance to other environmental stressors as homeostatic energy demands are increased to contend with contaminant stress (Liber *et al.* 1996; Sibley *et al.* 1997; McKenney *et al.* 1998).

The observed mixture effects on survival (Fig. 2) suggests that the IA model is the most suitable concept to predict the combined toxicity effects of the three chemicals tested in this study, likely due to the differences in modes of action of the assessed pesticides. Other studies have also found that the IA concept provides a reasonable prediction of toxic effect for mixtures containing compounds with different modes of action (including pyrethroids and organophosphates, Backhaus et al. 2000; Faust et al. 2003; de Zwart and Posthuma 2005; Barata et al. 2012). However, the CA model has been highlighted by others (Könemann 1981; Hermens *et al.* 1984; Cedergreen 2014; Chen *et al.* 2014) to better predict mixture toxicity (e.g., chlorpyrifos and esfenvalerate; Belden and Lydy (2006)), indicating that the IA under-predicts toxicity. Variable results between studies, which evaluated different types of mixtures containing organophosphates and pyrethroids, may have been due to the toxicity of the most toxic constituent, rather than their modes of action. In this study, lambda-cyhalothrin was 5-12 times more potent than permethrin and chlorpyrifos, respectively, and thus is likely the driver of toxicity in the mixtures. This supports previous postulations that CA and IA models can be driven by one chemical within mixtures if its potency is substantially higher than the other members of the mixture (Heindel et al. 1995; Olmstead and LeBlanc 2005).

Effects on sublethal endpoints of *C. dilutus* were observed at environmentally relevant concentrations. This was also found in a study by Maul *et al.* (2008) where the individual exposure to lambda-cyhalothrin or permethrin significantly affected immobilization and growth rate of *C. dilutus*. Due to the complex life cycle of *C. dilutus* (involving pupation and emergence events), larval growth is frequently demonstrated to be a more sensitive endpoint than survival during the larval period,

because growth may predict survival to adulthood and fecundity (Ankley *et al.* 1993; Ankley *et al.* 1994; Burton *et al.* 1996; Maul *et al.* 2008). Growth inhibition could lead to failure to mature and reproduce. In previous studies, reduced larval growth in *C. dilutus* negatively affected pupation, emergence (86 to 100% reduction), adult female size, number of eggs per female, and fecundity (Liber *et al.* 1996; Sibley *et al.* 1997; Ristola *et al.* 1999).

Agra and Soares (2009) also report that environmentally relevant concentrations of chlorpyrifos did not affect growth in *C. dilutus* supporting the lack of detectable effect on growth in this study. While daily food provision was equal throughout the tests, increased mortality resulting from exposure may have resulted in increased food availability *per capita*, thus concealing potential growth effects; as reported in other studies (Sibley *et al.* 1996; Martinez-Jeronimo *et al.* 2000; Hooper *et al.* 2003; Rakotondravelo *et al.* 2006). Furthermore, density can have negative effects on growth, development and reproduction, as reported by Hooper *et al.* (2003). But regardless of food *per capita* and density, growth was also affected by the pyrethroid pesticides, thus the effects observed following pyrethroid exposure, but not following exposure to chlorpyrifos, are potentially due to differences in mode of action of the two insecticide groups. Pyrethroids work by preventing closure of the sodium channels in neuronal membranes affecting both the peripheral and central nervous system which may directly lead to decrease in feeding activity (F. Landrum 2002), assimilation efficiency (Jager et al. 2006), protein synthesis, and rates of biotransformation and damage repair (Kooijman and Metz 1984). Chlorpyrifos can block the function of acetylcholinesterases (AChE), an important enzyme involved in neurotransmission. A number of studies found that despite a significant inhibition of AChE chironomid larvae are able to survive (Rakotondravelo *et al.* 2006; Rebechi *et al.* 2014), while behavioral effects such as limited mobility are present in organisms (Azevedo-Pereira *et al.* 2011) potentially having a lesser effect on feeding ability.

Mobility was the most sensitive endpoint in this study. A variety of neurotoxic contaminants in aquatic systems may affect mobility at sublethal exposure levels (Christensen *et al.* 2005; Werner and Moran 2008; Jin *et al.* 2009), thus suggesting to be a highly environmentally-relevant endpoint. It is especially useful for estimating effects on individual level in fish (Little and Finger 1990; Heath *et al.* 1993; Geist *et al.* 2007; Floyd *et al.* 2008; Beggel *et al.* 2010), and has also been applied in

experiments involving *C. dilutus* (Hatch and Burton 1999). The assessment of swimming performance incorporates biochemical and physiological effects and directly evaluates the impacts of neurotoxic contaminants on nerve cell transmissions and resulting muscle activity (Heath *et al.* 1993; Jin *et al.* 2009). Inability to swim normally after an exposure to insecticides will therefore negatively affect individual fitness and survival, with potential consequences at the population level (Little and Finger 1990; Floyd *et al.* 2008). In the current study, significant effects on mobility were detected at exposures that are within the range of reported environmental concentrations (Anderson *et al.* 2006; Budd *et al.* 2009; Werner *et al.* 2010; Weston and Lydy 2010).

In ecological risk assessment, safety factors are applied to account for the uncertainty of extrapolating from laboratory toxicity tests to the real environment (EC 2003; U.S.EPA 2004; Jager *et al.* 2006; Hanson and Stark 2012). Despite these regulation efforts, pesticides exceed sublethal effective concentrations (NOEC and EC50) determined for growth and immobility in this study (Amweg *et al.* 2006; Weston and Lydy 2010; Bereswill *et al.* 2013; Li *et al.* 2013), and have been shown to impact aquatic organisms worldwide at concentrations that current legislation considers environmentally protective (Werner *et al.* 2002; Schulz 2004; Weston and Lydy 2012; Beketov *et al.* 2013). Thus, it is evident that the toxicity of contaminants, and complex mixtures, may be significantly underestimated, by solely utilizing mortality as an endpoint for monitoring ambient water quality, as well as in ecological risk assessments, indicating the need for more sensitive endpoints to adequately assess ecological risk.

## 3.6 Conclusion

Determining the effect of water pollution remains a great challenge for environmental policy and management. This study reinforces the pressing need of integrating sublethal endpoints into regulatory toxicity assessments and monitoring studies to adequately assess the effects of contaminants. Mortality alone, as typically used for ecological assessment and management of industrial chemicals and pesticides, provides limited to no information on organism fitness caused by pesticide exposure, especially since environmentally relevant concentrations do not generally occur at concentrations that result in direct mortality to aquatic communities. The use of sublethal endpoints,

such as growth or mobility, in toxicity tests can indicate the presence of low levels of contaminants in water or sediment samples, sometimes at concentrations below the limit of detection of current-use analytical methods. Therefore it is essential to incorporate such tests into ambient water monitoring efforts and ecological risk assessment. In order to be able to monitor the effects of contaminant mixtures and to safeguard human health and the environment, a more holistic approach is required that includes assessing the combined effects of cumulative exposures to multiple stressors utilizing sublethal endpoints on toxicity exposures.

# 4. A Long-term Assessment of Pesticide Mixture Effects on Aquatic Invertebrate Communities

*A similar version of this chapter was submitted as: Simone Hasenbein, Sharon P. Lawler, Juergen Geist, Richard E. Connon. Environmental Toxicology and Chemistry. In Press.*

## 4.1 Abstract

To understand the potential effects of pesticide mixtures on aquatic ecosystems, studies that incorporate increased ecological relevance are crucial. Using outdoor mesocosms, we examined long-term effects of tertiary mixtures of two pyrethroid pesticides (permethrin, lambda-cyhalothrin), along with an organophosphate (chlorpyrifos) on aquatic invertebrate communities. Application scenarios were based on environmentally relevant concentrations (Env.Rel) and stepwise increases of lethal concentrations based on laboratory tests on *Hyalella azteca* and *Chironomus dilutus*, from LC 10 to LC50. Pyrethroids rapidly dissipated from the water column, while chlorpyrifos was detectable, even after six weeks of exposure. Twelve of fifteen macroinvertebrate and ten of sixteen zooplankton taxa responded significantly to contaminant exposures. The most sensitive taxa were the snail *Radix*, the amphipod *H. azteca*, the water flea *Daphnia magna*, and copepods. Env.Rel. had acute effects on *D. magna* and *H. azteca* (occurring 24h after application) while lag-times were more pronounced in *Radix* snails and copepods indicating chronic sublethal responses. Greatest effects on zooplankton communities were observed in Env.Rel treatments. Results indicate that insecticide mixtures continue to impact natural systems over a longer period of time, even when bound to particles and no longer detectable, causing consequences across multiple trophic levels due to a combination of indirect and direct effects.

## 4.2 Introduction

The increasing chemical pollution of freshwater systems worldwide, with largely unknown long-term effects on aquatic life and human health, is one of the key environmental challenges in the field of ecotoxicology (Vorosmarty *et al.* 2010). In areas of intense pesticide use, aquatic ecosystems and food webs are exposed to a number of chemicals that may alter species abundances and diversity (Butchart *et al.* 2010). Many of these compounds have been shown to cause negative effects even at low concentrations that current legislation considers environmentally protective (Beketov *et al.* 2013). Exposures to pesticides can result in reduced ecological fitness, and consequently impact survival of individual non-target species at different trophic levels through sublethal physiological, behavioral, or immunological effects, potentially leading to changes in structure and function of non-target populations affecting food web and ecosystem dynamics (Werner *et al.* 2010). As pesticide use continues to increase, there is a pressing need to understand how aquatic ecosystems respond to combinations of insecticides.

Although current risk-evaluation procedures that assess effects of individual pesticides on single species are needed for standardized comparisons of pesticide toxicities, these are typically performed at a narrow range of temperatures and environmental conditions and do not necessarily consider potential effects on different organism groups or interactions of trophic levels within aquatic ecosystems (Heugens *et al.* 2001). Additionally, waters within agricultural landscapes are often exposed to a variety of pesticides simultaneously and repeatedly, in differing combinations over time. Tests of chronic toxicity of pesticide mixtures in multi-species mesocosms represent a more ecologically relevant approach to ecotoxicological risk assessment that could aid ecosystem management efforts (Pereira *et al.* 2014).

Pyrethroids and organophosphates are neurotoxins with different neurological target sites and/or modes of action. Pyrethroids (lambda-cyhalothrin and permethrin) are nervous system toxicants that act primarily upon sodium channels in nerve cell membranes (Narahashi 2002). They slow the action of the sodium channel gates, causing them to be open for longer periods of time and resulting in hyperexcitation, tremors, convulsions, followed by lethargy and paralysis (Narahashi 2002).

Chlorpyrifos, an organophosphate, inhibits acetylcholine esterase activity directly impacting the synaptic signal which leads to an accumulation of acetylcholine and subsequent impairment of numerous body functions (Malison *et al.* 2010). We selected these compounds because they are among the most frequently detected insecticides in aquatic habitats worldwide (Amweg *et al.* 2006), they are known to be highly toxic to non-target organisms (Werner *et al.* 2010), and they are used in agriculture and landscape maintenance. Thus, they are repeatedly detected as mixtures in the same water and sediment samples at concentrations that are known to be toxic to aquatic life (Weston *et al.* 2014).

By using outdoor mesocosms, we investigated the long-term effects on invertebrate communities of mixtures of the insecticides lambda-cyhalothrin, permethrin, and chlorpyrifos. Given that aquatic communities may respond differently to exposure to pesticide mixtures compared to results of laboratory risk assessments, an outdoor mesocosm approach is arguably a more realistic approach to evaluating effects on aquatic communities. By assessing the long-term effects of multiple pesticide mixture applications at concentrations typically found in the environment and known to cause toxic effects in laboratory tests, we evaluated the ecological risk of pesticide mixtures, and the usefulness of mesocosm systems for understanding long-term effects.

## 4.3 Material and Methods

*Experimental Systems*

The mesocosm system used is located at the Putah Creek Riparian Reserve (University of California at Davis, CA, USA). The experimental setup consisted of sixteen polyethylene plastic tanks (Loomis Tank Centers, Jackson, CA, USA), each with a length and width of 185.4 cm, a height of 64.8 cm, and a volume of 1325 L. To eliminate possible contamination from the manufacturing process, each tank was leached in water and exposed to weather conditions (UV light, rain) for one year, before the start of experiments. Tanks were buried 30 cm into the ground for natural temperature buffering.

Before adding sediment and water to each tank, toxicity was assessed by conducting two separate 96h sediment, and water toxicity tests using the amphipod *Hyalella azteca* (U.S.EPA 2000). No mortality was observed in either test (data not shown). Each tank was filled with a 10-cm layer of sandy-loam mixture as substrate. The sediment mixture consisted of 50% sand to avoid eutrophication of tank water (ACE Hardware, Oak Brook, IL, USA). The loam was taken from a nearby pond within the reserve; neither the pond nor its surroundings are treated with pesticides or herbicides. The loam contained benthic invertebrates that were used to inoculate the mesocosm substrate with naturally derived organisms. Water was drawn from the same pond to provide algae and bacteria. Tanks were filled to a depth of 50 cm. Due to evaporation, all tanks were refilled with filtered pond water if any of the tanks' water level dropped below 45 cm. Equal numbers of macrophytes were added to each tank: five individual plants (10 cm long sprig each) of the pondweed *Elodea canadensis* and of the whorled water-milfoil *Myriophyllum verticillatum*, both obtained from a nearby creek. Once the system was installed, tanks were left from February to May 2013 to stabilize and to allow immigration of flying aquatic taxa (e.g, Odonata, Ephemeroptera, Hemiptera, Coleoptera). Such immigration continued throughout the course of the study, promoting an intact community, and supporting recovery by recolonization following pesticide application.

*Pesticide Application*

Commercially available pesticide formulations of permethrin (Pounce 25 WP™), lambda-cyhalothrin (Warrior II™), and chlorpyrifos (Lorsban-4E™) were used to make treatment solutions (Table 7).

Table 7 Pesticide formulations used in this study including active ingredients, pesticide class (type), relative purities, and Chemical Abstracts Service (CAS) Number.

| Trade name | Active ingredient | Active Ingredient (%) | Type | CAS Number |
|---|---|---|---|---|
| Pounce 25 WP | Permethrin | 25.0 | Pyr I | 52645-53-1 |
| Warrior II | Lambda-cyhalothrin | 22.8 | Pyr II | 91465-08-6 |
| Lorsban-4E | Chlorpyrifos | 44.9 | OP | 5598-13-0 |

Pyr I = pyrethroid type I, Pyr II = pyrethroid type II, OP = organophosphate.

Controls and pesticide treatments were randomly assigned to the tanks, with four replicates each. Five application events were conducted on 06/19/2013 (week 0), 08/08/2013 (week 7), 09/17/2013 (week 13), 10/01/2013 (week 15), and 10/15/2013 (week 17). Treatment solutions were made by dissolving each pesticide formulation in de-ionized (DI) water (stock solution) and then spiking each stock solution into 1 L DI water to achieve corresponding nominal concentrations for each pesticide mixture.

Treatment solutions (50 mL per tank) were applied with a handheld sprayer (1.8 L volume, Root-Lowell Flo-Master®, Lowell, MI, USA), distributing the treatment solution evenly on water surfaces. To avoid drift, applications were only conducted when no wind was present and control tanks were covered with plastic sheets during each application event. Plastic sheets were removed once the application was completed. Three treatments and a control were used to investigate the mixture effects on the invertebrate community. These three treatments were based on known environmentally relevant concentrations (Env.Rel.) or lethal concentrations (LC) determined in previous laboratory studies using *H. azteca* (LC-Hya) and *C. dilutus* (LC-Chiro) (Table 8) (Hasenbein *et al.* 2015).

Table 8 Nominal (N) and measured (M) concentrations of each application event conducted in weeks 0, 7, 13, 15, and 17 for the environmentally relevant (Env.Rel.) and lethal concentrations (LC) treatments in ng/L for each pesticide (N = 4). Pesticides were applied in tertiary mixtures. Lethal concentrations were determined in previous laboratory studies investigating the tertiary mixture effects on *H. azteca* (LC-Hya) and *C. dilutus* (LC-Chiro). Application concentrations of Env.Rel. were the same across all five application events, while LC-Hya and LC-Chiro increased from LC10 to LC50.

| | | Pesticide concentration (ng/L) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Week | | 0 | | | 7 | | | 13 | | | 15 | | | 17 | | |
| | | N | M | SE | N | M | SE | N | M | SE | N | M | SE | N | M | SE |
| Env.Rel. | CLF | 7.50 | 7.36 | 1.47 | 7.50 | 5.63 | 0.62 | 7.50 | 5.48 | 0.99 | 7.50 | 9.02 | 1.96 | 7.50 | 12.86 | 4.76 |
| | LC | 3.50 | 2.78 | 0.05 | 3.50 | 3.59 | 0.05 | 3.50 | 4.15 | 0.65 | 3.50 | 5.46 | 1.29 | 3.50 | 8.00 | 2.81 |
| | Perm | 5.70 | 6.60 | 0.73 | 5.70 | 10.69 | 5.35 | 5.70 | 7.50 | 4.10 | 5.70 | 8.63 | 7.48 | 5.70 | 13.13 | 11.38 |
| LC-Hya | CLF | 58.10 | 51.18 | 2.37 | 66.95 | 59.37 | 2.87 | 66.95 | 70.78 | 10.75 | 77.15 | 66.46 | 26.17 | 77.15 | 75.06 | 16.38 |
| | LC | 0.14 | 0.70 | 0.20 | 0.17 | 1.21 | 0.35 | 0.17 | 1.93 | 0.32 | 0.21 | 2.54 | 0.81 | 0.21 | 3.28 | 0.96 |
| | Perm | 48.56 | 43.99 | 4.74 | 55.01 | 56.06 | 2.10 | 55.01 | 66.63 | 16.15 | 62.30 | 71.63 | 15.91 | 62.30 | 77.63 | 0.96 |
| LC-Chiro | CLF | 161.78 | 137.92 | 14.83 | 284.41 | 264.80 | 31.02 | 284.41 | 323.31 | 126.45 | 522.97 | 326.34 | 48.44 | 522.97 | 384.06 | 93.71 |
| | LC | 37.78 | 30.10 | 2.97 | 43.31 | 49.90 | 2.13 | 43.31 | 47.58 | 10.62 | 49.65 | 58.85 | 0.47 | 49.65 | 56.16 | 7.62 |
| | Perm | 128.52 | 115.47 | 16.21 | 192.07 | 187.53 | 35.75 | 192.07 | 188.26 | 44.42 | 267.11 | 266.50 | 28.47 | 267.11 | 229.05 | 111.98 |

Perm = permethrin, LC = lambda-cyhalothrin, CLF = chlorpyrifos. N = nominal concentration, M = measured concentration, SE =standard error

Pesticide concentrations used for Env.Rel. were equal for all application events to imitate a steady low-level contamination of a freshwater aquatic ecosystem. However, concentrations for LC-Hya and LC-Chiro increased over time as to first monitor any sublethal long-term responses caused by low pesticide concentrations and then imitate acute ecological relevant run-off events that may impact the ecosystem within shorter periods of time. Thus, the first application for these two treatments was based on LC10 values (LC10-Hya, LC10-Chiro), the second and the third application on LC25 (LC25-Hya, LC25-Chiro), and the fourth and fifth application on LC50 (LC50-Hya, LC50-Chiro) (Table 8).

*Sampling Procedure*

Sampling was carried out weekly starting six weeks before the first pesticide application on June 19$^{th}$, 2013 (week 0) to examine system stability, and ended on October 31$^{st}$, 2013 (week 19) resulting in a total sampling period of 25 weeks. At each sampling day, physicochemical parameters (water temperature, pH, dissolved oxygen and specific conductance) were measured in each tank before biological sampling.

Pelagic and benthic macroinvertebrate species were sampled by using an aquatic D-net (mesh size = 150 µm, 30.48 cm in diameter, total net area = 510 cm$^2$, BioQuip Products, Inc., Rancho Dominguez, CA, USA) and artificial habitat samplers. Separate D-nets were used for the control and the treated tanks to avoid contamination of the control. Netting was conducted by sweeping along each side and through the center of each tank in a bottom-to-surface motion resulting in 283.50 L of total water volume sampled, a standardized procedure to capture pelagic organisms and any organism clinging to the tank wall or plants. Artificial habitat samplers were built based on Brock *et al.* (1992) and consisted of a wire frame (20 x 20 x 5 cm; mesh size = 1.2 x 1.2 cm), filled with pebbles (diameter of 32 to 64mm), and five hardwood plates (10 x 10 x 0.4 cm) screwed to a stainless steel pin (length = 1m, diameter = 0.5 cm). Each sampler was placed on a rectangular screen (73.5 x 73.5 cm, mesh size = 150 µm) that was tied to a 125 mL PVC bottle (without the lid). By ensuring that the screen was flat on the ground, organisms were able to enter and leave the habitat samplers. By pulling the PVC bottle up the screen wrapped around the habitat sampler and prevented organisms from

escaping. Two habitat samplers were used per tank and sampled in alternate weeks, allowing a colonization time of two weeks for each sampler. Organisms caught by the netting or habitat samplers were rinsed into a white tray, identified on-site, and transferred back into the corresponding tank as to not to interfere with the development of the macroinvertebrate community. Organismal counts are presented as the sum of both the netting and the habitat sampler counts per liter.

For zooplankton identification, a combined sample of four sub-samples per mesocosm tank was taken using a PVC tube (diameter = 4.8cm, length = 1m) and a stopper, using different tubes for each treatment and the controls. The tube was gently lowered to avoid sediment perturbation. Based on water depth, the total water volume taken was calculated. Collected water (~ 2.8 L total volume) was poured through a stainless steel sieve (pore size = 63µm, diameter = 30.48 cm), organisms were rinsed into 125 mL Polyethylene-bottles, preserved with 90% ethanol, and stored at room temperature in the dark. The amount of water removed from each tank represented approximately 0.2% of the total water volume and thus was not expected to interfere with the overall zooplankton community development. At least one day before identification, samples were stained using a solution of rose bengal (Fisher Scientific, Waltham, MA, USA) dissolved in 70% Ethanol (Fisher Scientific, Waltham, MA, USA). Species were identified and counted using a stereoscopic microscope at 40-70x magnification. Cladocera and Rotifera were counted and identified to the lowest practical taxonomic level (genus-species), whereas copepods were counted and classified as calanoids and cyclopoids. Zooplankton abundances were expressed as numbers of organisms per liter.

Sweep-netting of macroinvertebrates was conducted immediately before zooplankton sampling which helped to distribute aggregations of zooplankton; this likely decreased sample variance. Habitat samplers were collected last. All organisms were counted and identified to the lowest practical taxonomic level.

To monitor effects on emergence of flying taxa, a floating emergence trap was positioned onto the water surface of each tank and sampled twice per week. Each trap was shaped like a four-sided pyramid with a base of 60 cm x 60 cm. The side frames were constructed of polyvinylchloride (PVC) pipes and were covered with a durable fine-mesh netting (mesh size = 200 µm)(Malison *et al.* 2010).

Traps were taken out before each pesticide application and placed back after 24h to minimize binding of the pesticides to the trap material.

*Chemical Analysis*

Water samples were taken 5cm below the water surface 1h after each application and weekly thereafter. Samples were collected using 950 mL amber pre-labeled and kilned glass bottles. Sediment samples were taken weekly from week 1 to 10, and then every other week thereafter. Sediment samples were collected from the top 2 cm using pre-cleaned stainless steel spoons and carefully transferred to 200-ml amber pre-labeled, kilned glass bottles. Both sampling procedures represent a water and sediment removal of less than 1% per sampling day to minimize any impact on the overall invertebrate community development. Water and sediment samples were transported on wet ice to the laboratory, stored in the dark at 4°C and extracted within two days of collection.

Water samples were spiked with trans-permethrin (dimethyl D6, EQ Laboratories, Atlanta, GA, USA) as a recovery surrogate and extracted using solid phase extraction cartridges (Supelclean ENVI™ - C18, 500 mg, Sigma-Aldrich, St. Louis, MO, USA). Cartridges were pre-conditioned using 12 mL ethyl acetate:hexane (50:50, v/v), 12 mL methanol, and 12 mL MilliQ water (EMD Millipore, Billerica, MA, USA). Samples were loaded onto the cartridge and eluted with 10 mL 1:1 ethyl acetate:hexane and evaporated to 0.4 mL at 40° under a gentle stream of nitrogen using a Turbovap (Biotage, Charlotte, NC, USA).

On clean aluminum dishes, 20g of wet sediment was dried overnight at 60ºC. An aliquot of 10g of dried and finely ground sediment was extracted three times in 50mL centrifuge vials using a total volume of 40 mL of dichloromethane:hexane (70:30, v/v) by sonicating for 30 min and centrifuging at 3000 rpm for 5 min for each extraction step. Twenty milliliters of the combined extracts were concentrated to 0.4 ml. To remove plant pigments such as chlorophyll and plant sterols without the loss of planar compounds, preconditioned GCB/PSA cartridges were used for a clean-up step (Supelclean™ ENVI-Carb™ II, Sigma-Aldrich). Cartridges were conditioned with 10 mL acetonitrile, 10 mL dicholoromethane, and 10 mL hexane. Extracts were loaded onto the cartridges, eluted with 7

ml of dichloromethane:hexane (70:30, v/v), and concentrated to 0.4 ml at 40° under a gentle stream of nitrogen using a Turbovap (Biotage).

The internal standard 4,4' dibromo-octafluorobiphenyl (Chem Service, West Chester, PA, USA) was added to all concentrated water and sediment extracts in order to correct quantitative differences in extract volume as well as to monitor instrument conditions. Extracts were analyzed using an HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an HP-5973N quadrupole mass spectrometer detector operated in electron capture negative ionization mode (GC-ECNI-MS) with methane as the reagent gas. The gas chromatograph was equipped with a Supelco DB-5MS (30 m x 0.25 mm with a 0.25 µm film thickness) with Helium as the carrier gas. A 1-µL of sample was injected in splitless mode (injector temperature 280°C, purge time 1.5 min). Instrumental calibration was performed using nine sets of calibration standard solutions containing all three pesticides (each purchased as 100 µg/ml solution in acetonitrile, Chem Service), the surrogate trans-permethrin D6 (EQ Laboratories, Atlanta, GA, USA), and the internal standard 4,4' dibromo-octafluorobiphenyl in hexane. Quantification of the pesticides was done based on peak areas and comparing them with the calibration curve normalized to the internal standard response. All calibration curves had an $r^2 > 0.99$.

Quality-assurance/quality-control was conducted by analyzing a method blank of deionized water (Milli-Q) to ensure that no contamination occurred during sample extraction and analysis. The surrogate trans-permethrin D6 was added to each sample, including the blank, before extraction to monitor matrix effects and overall method performance. Instrumental limit of detections (whole water) were as follows: 0.6 ng/L bifenthrin, 4.8 ng/L permethrin, 1.4 ng/L cyfluthrin, and 0.8 ng/L chlorpyrifos. Surrogate recoveries were on average 103% with a range between 60 – 120%. Reported values were not corrected for surrogate recovery. No pesticides were detected in the controls or the method blank.

*Data Analysis*

Physicochemical parameters and biological response variables were analyzed using repeated measures analysis of variance (RM-ANOVA). When a significant time X treatment interaction was

detected, one-way ANOVA followed by a Dunnett's multiple comparison was used to determine significant differences between treatments and controls within dates. Omega-squared ($\omega^2$) was calculated to assess the magnitude of an effect with 0.01, 0.06, and 0.14 representing a small, medium, and large effect size, respectively (Graham and Edwards 2001).

Levene's test and the Shapiro-Wilk test were used to test variance homogeneity and normality. When data was not normally distributed, ln-transformation was applied to achieve normality. If normality was still not achieved, but homogeneity of variances was met, a Kruskal-Wallis test was applied. All tests were carried out using Minitab 16 Statistical Software 2010 (Minitab, Inc., State College, PA, USA) with a significance level at $\alpha = 0.05$. All differences discussed below are significant unless otherwise noted.

Analysis of community structure was performed using the Principal Response Curves method (PRC), with Canoco 5 (Microcomputer Power, Ithaca, NY, USA) (Braak and Šmilauer 2002). All data were normalized and log (2x + 1) transformed prior to analysis. PRC uses dimension reduction to summarize all information on the investigated populations simultaneously and emphasizes the percentage change of abundance of a species relative to the control, independent from its absolute abundance, elucidating effects and impact of contaminants at the community level.

On the Y-axis, the PRC graph shows the canonical coefficients (C*dt*) which quantify the changes in taxa composition between the treatments and control (represented by X-axis) over time. The significance of the PRC diagram was tested by Monte Carlo permutation tests, using an *F*-type test statistic based on the eigenvalue of the component (preventing random results, $\alpha = 0.05$). The accompanying "species scores" reflect the influence of particular species on the overall community response described by the PRC over time (Van Den Brink *et al.* 1995). Species with high positive scores are positively correlated, species with negative scores response oppositely. Taxa are regarded important for the community reaction towards the treatment if their species score is higher than 0.5 (absolute value). Taxa with near zero scores are indifferent to the trends recognized by the PRC (Braak and Šmilauer 2002).

## 4.4 Results

*Physicochemical Parameters*

Mean temperature in the mesocosm tanks was 16.1 °C (±0.19 SE) over the entire sampling period (May through October), with a maximum temperature of 22.2°C (end of June through July) and a minimum temperature of 7.1 °C (end of October) (Figure 11A). Mean specific conductance was 595.6 µS/cm (±12.65 SE), dissolved oxygen 77.8% (±0.76 SE) and 7.7 mg/L (±0.07 SE, Figure 11B), and pH 9.6 (±0.02 SE, Figure 11C). RM-ANOVA detected a treatment effect on pH ($P = 0.001$, $F_{3,263} = 6.35$, $\omega^2 = 0.02$, Appendix A). One-way ANOVA showed a decrease in pH in week 8 (one week after 2nd application) and 9 (two weeks after 2nd application) for LC-Chiro ($P = 0.033$, $F_{3,12} = 4.07$, $\omega^2 = 0.36$, and $P = 0.025$, $F_{3,12} = 4.48$, $\omega^2 = 0.39$, respectively), and an increase in week 17 (48h after the fifth application) in LC-Hya ($P = 0.036$, $F_{3,12} = 3.95$, $\omega^2 = 0.36$) compared to the controls (Figure 11C).

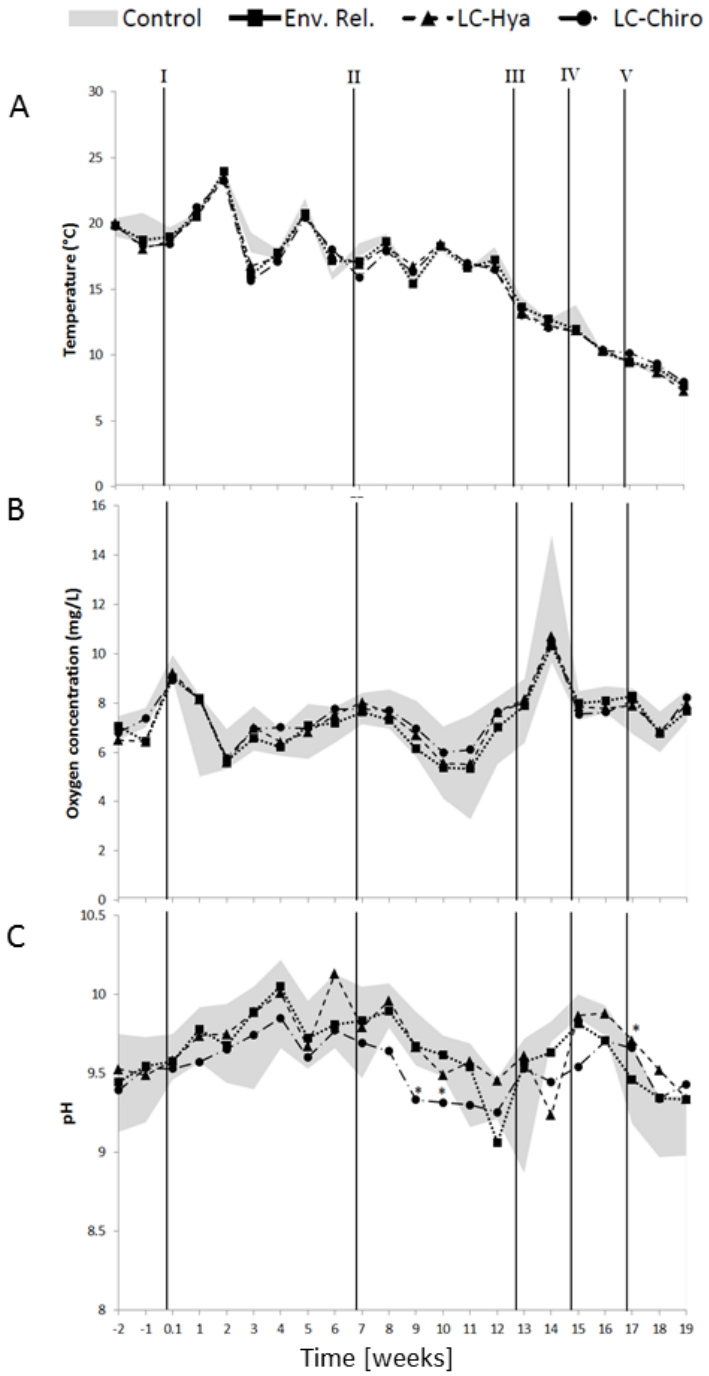


Figure 11 Change in temperature (A), oxygen concentrations (mg/L, B), and pH (C) for each treatment compared to the range of control values (grey area) over the course of the study period. X-axis = time [weeks], Y-axis = individuals/sample. Asterisk represents a statistically significant difference of the treatment relative to the control ($\alpha = 0.05$). Env.Rel. = environmentally relevant concentrations, LC-Hya and LC-Chiro = lethal concentrations derived from previous laboratory assessments using *H. azteca* (LC-Hya) and *C. dilutus* (LC-Chiro). Vertical lines represent application events (at 0, 7, 13, 15, and 17 weeks past first application). Application concentrations for LC-Hya and LC-Chiro increased over time: I = LC10, II and III = LC25, IV and V = LC50

*Pesticide Fate*

Measured application concentrations for each treatment differed from nominal target concentrations (Table 8). In Env.Rel., average measured application concentrations were 5.1 ng/L (±0.6 SE) chlorpyrifos, 2.1 ng/L (±0.5 SE) lambda-cyhalothrin, and 3.3 ng/L (±1.1 SE) permethrin across the entire study period. Concentrations in LC-Hya and LC-Chiro were increased over time (Figure 12A). In LC-Hya, application concentrations were measured from 51.2 ng/L (±2.4 SE) chlorpyrifos, 0.7 ng/L (±0.2 SE) lambda-cyhalothrin, and 43.9 ng/L (±4.7 SE) permethrin to 75.1 ng/L (±16.4 SE) chlorpyrifos, 3.3 ng/L (±1.0 SE) lambda-cyhalothrin, and 77.6 ng/L (±14.5 SE) permethrin (Figure 12B). In LC-Chiro, application concentrations were measured from 137.9 ng/L (±14.8 SE) chlorpyrifos, 30.1 ng/L (±2.9 SE) lambda-cyhalothrin, and 115.5 ng/L (±16.2 SE) permethrin to 384.1 ng/L (±93.7 SE) chlorpyrifos, 56.2 ng/L (±7.6 SE) lambda-cyhalothrin, and 229.1 ng/L (±112.0 SE) permethrin (Figure 12C).

Across all treatments, permethrin dissipated the fastest from the water column and was not detected within one to four weeks following pesticide applications, indicating an average dissipation rate of 56.9% (±6.9 SE) per week (Figure 12A-C). Lambda-cyhalothrin dissipated from the water column between two and six weeks following an application (average weekly dissipation rate = 39.8%, ±6.7 SE). Chlorpyrifos concentrations declined on average 32.0% (±6.0 SE) per week representing the slowest dissipation rate of the three chemicals measured.

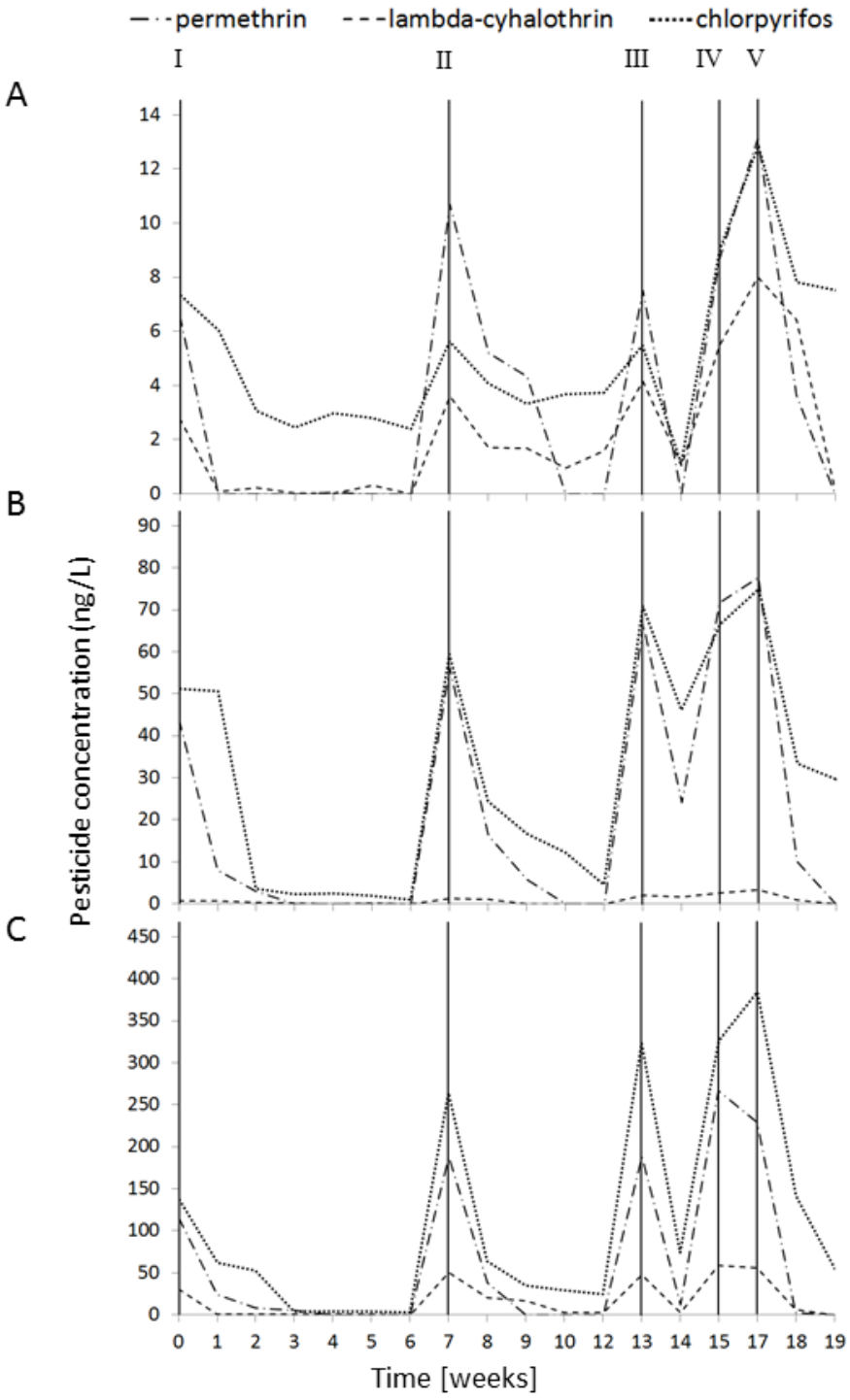


Figure 12 Average water concentrations (ng/L) of permethrin, lambda-cyhalothrin, and chlorpyrifos per treatment. (A) = Env.Rel., (B) = LC-Hya, (C) = LC-Chiro. Sampling was conducted weekly, except for week 11 and 16 when no samples were taken. No pesticides were detected in control tanks, and are thus not presented herein. X-axis = time (weeks). Vertical lines represent application events at 0, 7, 13, 15, and 17 weeks. Application concentrations for LC-Hya and LC-Chiro increased over time: I = LC10, II and III = LC25, IV and V = LC50

Concentrations of sediment samples in Env.Rel. were on average 0.47 ng/L (±0.10 SE) chlorpyrifos, 0.68 ng/L (±0.19 SE), and 0.19 ng/L (±0.14 SE) permethrin. Maximum concentrations never exceeded 7 ng/L for all chemicals (Figure 13A). The highest concentrations for each pesticide were measured in week 9 (5.96 ng/L lambda-cyhalothrin and 6.89 ng/L permethrin) and week 10 (3.19 ng/L chlorpyrifos). In LC-Hya, sediment concentrations were on average 0.31 ng/L (±0.06 SE) ng/L chlorpyrifos, 0.71 ng/L (±0.18 SE) lambda-cyhalothrin, and 1.16 ng/L (±0.67 SE) permethrin (Figure 13B). Maximum concentrations measured for each pesticide were 1.52 ng/L chlorpyrifos (week 6), 4.62 ng/L lambda-cyhalothrin (week 10), and 29.94 ng/L permethrin (week 5). In LC-Chiro, average concentrations in the sediment were measured at 1.24 ng/L (±0.86 SE) for chlorpyrifos, 0.81 ng/L (±0.43 SE) lambda-cyhalothrin, and 0.44 ng/L (±0.34 SE) permethrin (Figure 13C).

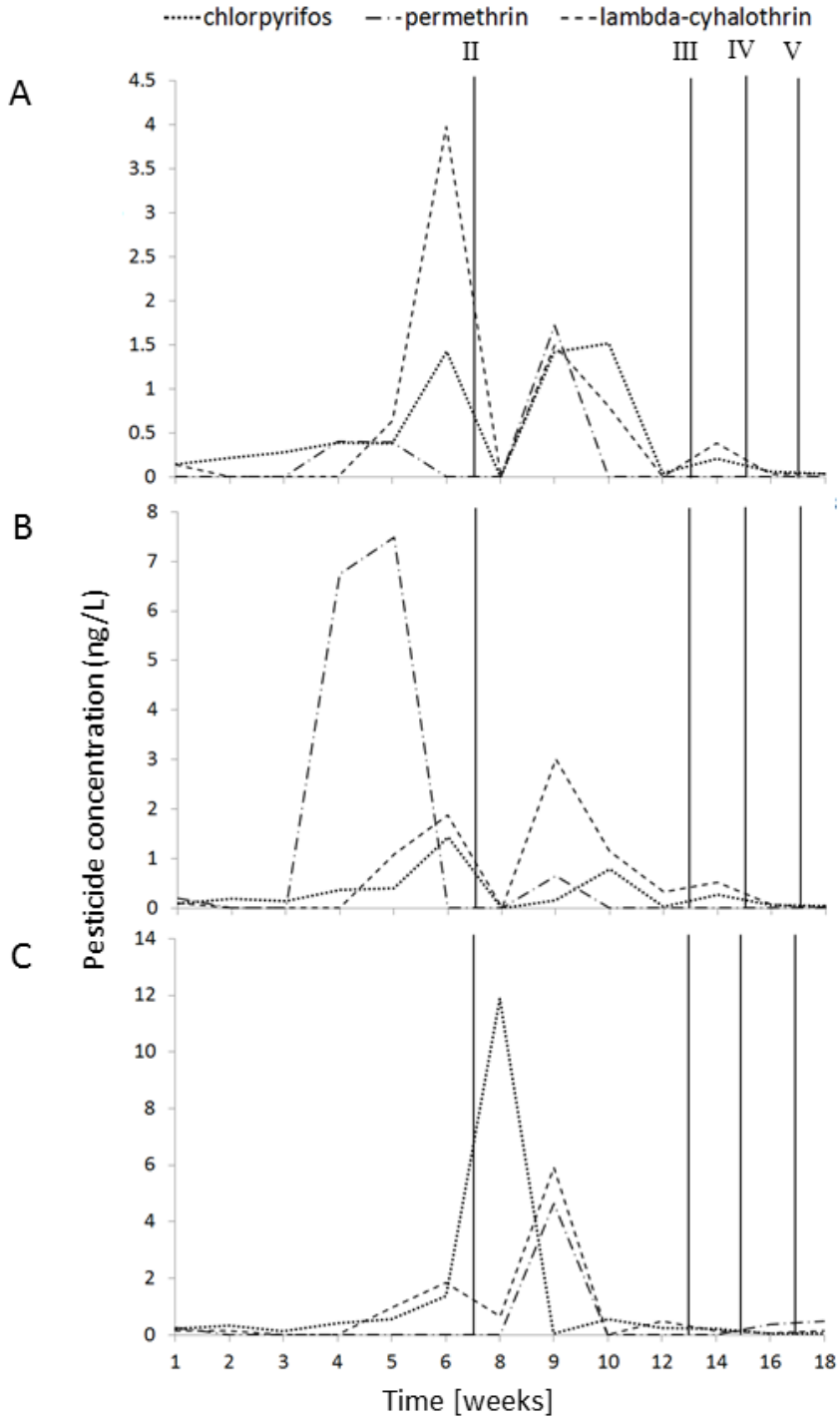


Figure 13 Average sediment concentrations (ng/L) of permethrin, lambda-cyhalothrin, and chlorpyrifos per treatment. (A) = Env.Rel., (B) = LC-Hya, (C) = LC-Chiro. Sampling was conducted weekly until week 10, and then every other week thereafter. No pesticides were detected in control tanks, and are thus not presented herein. X-axis = time (weeks). Vertical lines represent application events at 0, 7, 13, 15, and 17 weeks. Sediment sampling started one week following the first application, thus first application event is not visible in this graph. Application concentrations for LC-Hya and LC-Chiro increased over time: I = LC10, II and III = LC25, IV and V = LC50

*Macroinvertebrate Response*

Twenty-five macroinvertebrate taxa were identified in the mesocosms. Most taxa were in the order Diptera (8 taxa), followed by Coleoptera (5 taxa), Heteroptera (3 taxa), Pulmonata and Odonata (2 taxa each). Hirudinea, Amphipoda, Hydracarina, Annelida, and Turbellaria were represented with one taxon each. Summed over the study period, Zygoptera (damselflies) represented the largest portion of macroinvertebrate abundance (20.9%), followed by the pulmonate snail *Radix* sp. (14.8%), and the amphipod *H. azteca* (13.2%).

Pesticide exposure affected abundances of twelve out of fifteen analyzed macroinvertebrate taxa. Overall treatment effects combine pesticide levels, but reveal which taxa are abundant and sensitive enough to show effects. RM-ANOVA of combined data from sweep netting and habitat samplers showed treatment effects from greatest to lowest sensitivity (in order of F-value herein, Table 9), for the orders Amphipoda (*Hyalella azteca* was the only representative), Ephemeroptera, Pulmonata, Odonata, Diptera, Coleoptera. Between the two Odonata suborders, the chemical exposure had a greater effect on Anisoptera than Zygoptera. Within the Pulmonata, effects on the Planorbidae were detectable but weak. Abundant genera were also analyzed individually, and RM-ANOVA revealed treatment effects from greatest to smallest, on *Anopheles*, *Radix*, and *Culex*.

Treatment effects varied over time for some taxa (time X treatment interaction) and resulted in the following order from greatest to lowest sensitvity: *Anopheles* sp., *H. azteca*, *Culex* sp., Diptera, Zygoptera, and gill-breathing insects (Table 9 and Appendix A). For Heteroptera, Hirudinea, Hydrocarina, Annelida, and Turbellaria, insufficient sample sizes were recorded (≤ 12 per sampling day across all tanks), thus statistical analysis was not feasible.

Table 9 Results of repeated measures ANOVA testing the effect of treatment and the time versus treatment interaction on abundances of physicochemical parameters, abundances of macroinvertebrate taxa, and emergence of macroinvertebrate taxa. Only taxa with significant P-values are listed herein.

| | | d.f. | F | P | $\omega^2$ |
|---|---|---|---|---|---|
| *Macroinvertebrates* | | | | | |
| | Odonata | | | | |
| | Treatment | 3 | 4.34 | 0.008 | 0.02 |
| | Error | 264 | | | |
| | Zygoptera | | | | |
| | Treatment | 3 | 6.57 | 0.001 | 0.04 |
| | Time x Treat. | 63 | 1.4 | 0.037 | 0.04 |
| | Error | 256 | | | |
| | Anisoptera | | | | |
| | Treatment | 3 | 8.46 | < 0.001 | 0.05 |
| | Error | 244 | | | |
| | Diptera | | | | |
| | Treatment | 3 | 3.09 | 0.033 | 0.02 |
| | Time x Treat. | 63 | 1.47 | 0.019 | 0.05 |
| | Error | 264 | | | |
| | Ephemeroptera | | | | |
| | Treatment | 3 | 6.14 | 0.001 | 0.00 |
| | Error | 264 | | | |
| | *Anopheles* sp. | | | | |
| | Treatment | 3 | 4.51 | 0.004 | 0.03 |
| | Time x Treat.[1] | 60 | 1.61 | 0.008 | 0.07 |
| | Error | 249 | | | |
| | *Culex* sp. | | | | |
| | Treatment | 3 | 3.11 | 0.033 | 0.02 |
| | Time x Treat. | 63 | 1.52 | 0.013 | 0.06 |
| | Error | 264 | | | |
| | Coleoptera | | | | |
| | Treatment | 3 | 3.06 | 0.035 | 0.01 |
| | Error | 264 | | | |
| | *H. azteca* | | | | |
| | Treatment | 3 | 21.17 | < 0.001 | 0.11 |
| | Time x Treat. | 63 | 1.56 | 0.015 | -0.01 |
| | Error | 154 | | | |
| | Pulmonata | | | | |
| | Treatment | 3 | 5.03 | 0.003 | 0.00 |
| | Error | 264 | | | |
| | Planorbidae | | | | |
| | Treatment | 3 | 4.51 | 0.006 | 0.00 |
| | Error | 264 | | | |
| | *Radix* sp. | | | | |
| | Treatment | 3 | 3.51 | 0.02 | 0.01 |
| | Error | 283 | | | |
| *Emergence* | | | | | |
| | Zygoptera | | | | |
| | Treatment | 3 | 3.07 | 0.037 | 0.01 |
| | Error | 204 | | | |

[1] = Lack-of-fit, d.f. = degrees of freedom, F = F-ratio, P = P-value. $\omega^2$ = Magnitude of effects.

The environmentally-relevant treatment (Env.Rel.) had the earliest and most consistently negative effects on most taxa, followed by LC-Chiro, and the weakest effects were observed in LC-Hya. Results following are from one-way ANOVA Dunnett's test and Kruskal-Wallis tests for significant RM-ANOVA results, ordered from most sensitive to least sensitive. The most sensitive macroinvertebrate species was *H. azteca*, which responded with a negative trend in weeks 9 – 15 in

Env.Rel. and LC-Chiro (P = 0.140 – 0.404, $F_{3,7}$ = 1.15 – 2.54, $\omega^2$ = 0.04 – 0.30), followed by negative effects in both treatments in weeks 16 to 19 (P = 0.006 - 0.033, $F_{3,7}$ = 5.24 – 9.96, $\omega^2$ = 0.54 – 0.71) displaying the greatest magnitude of effect ($\omega^2$) and long-term effects on abundance of the macroinvertebrate taxa (Figure 14A). *Radix* abundance in Env.Rel., LC-Hya, and LC-Chiro was decreased in weeks 10, 11, 12, and 17 (P = 0.001 – 0.005, $F_{3,10}$ = 8.05 – 12.36, $\omega^2$ = 0.60 – 0.71), representing the second most affected macroinvertebrate species (Figure 14B). Zygoptera was the third most sensitive taxon and displayed a decreased abundance in LC-Chiro in weeks 2 (P = 0.043, d.f. = 1, H = 4.08), 8 (P = 0.043, d.f. = 1, H = 4.08), 16, 17 (both: P = 0.021, d.f. = 1, H = 5.33), and 18 (P = 0.035, $F_{3,12}$ = 3.99, $\omega^2$ = 0.36) (Figure 14C). The overall abundance of Odonata decreased in LC-Chiro in weeks 17 (P = 0.021, d.f. = 1, H = 5.33), 18 (P = 0.032, $F_{3,12}$ = 4.13, $\omega^2$ = 0.37), and 19 (P = 0.029, $F_{3,12}$ = 4.27, $\omega^2$ = 0.38). Anisoptera abundance did not significantly deviate from the control over the course of the study period, but displayed a positive trend in all treatments from week 2 to 5 (P = 0.146 – 0.149, d.f. = 1, H = 2.08 – 2.11). This trend was not visible in the Odonata abundance possibly because Anisoptera and Zygoptera responded in opposite directions. Taxa responding with positive effects were *Anopheles* sp. which displayed an increased abundance in Env.Rel. in weeks 2 (P < 0.001, $F_{3,11}$ = 14.75, $\omega^2$ = 0.73) and 10 (P = 0.043, d.f. = 1, H = 4.08), and gill-breathing insects (increase in abundance in week , P = 0.015, $F_{3,12}$ = 5.31, $\omega^2$ = 0.45).

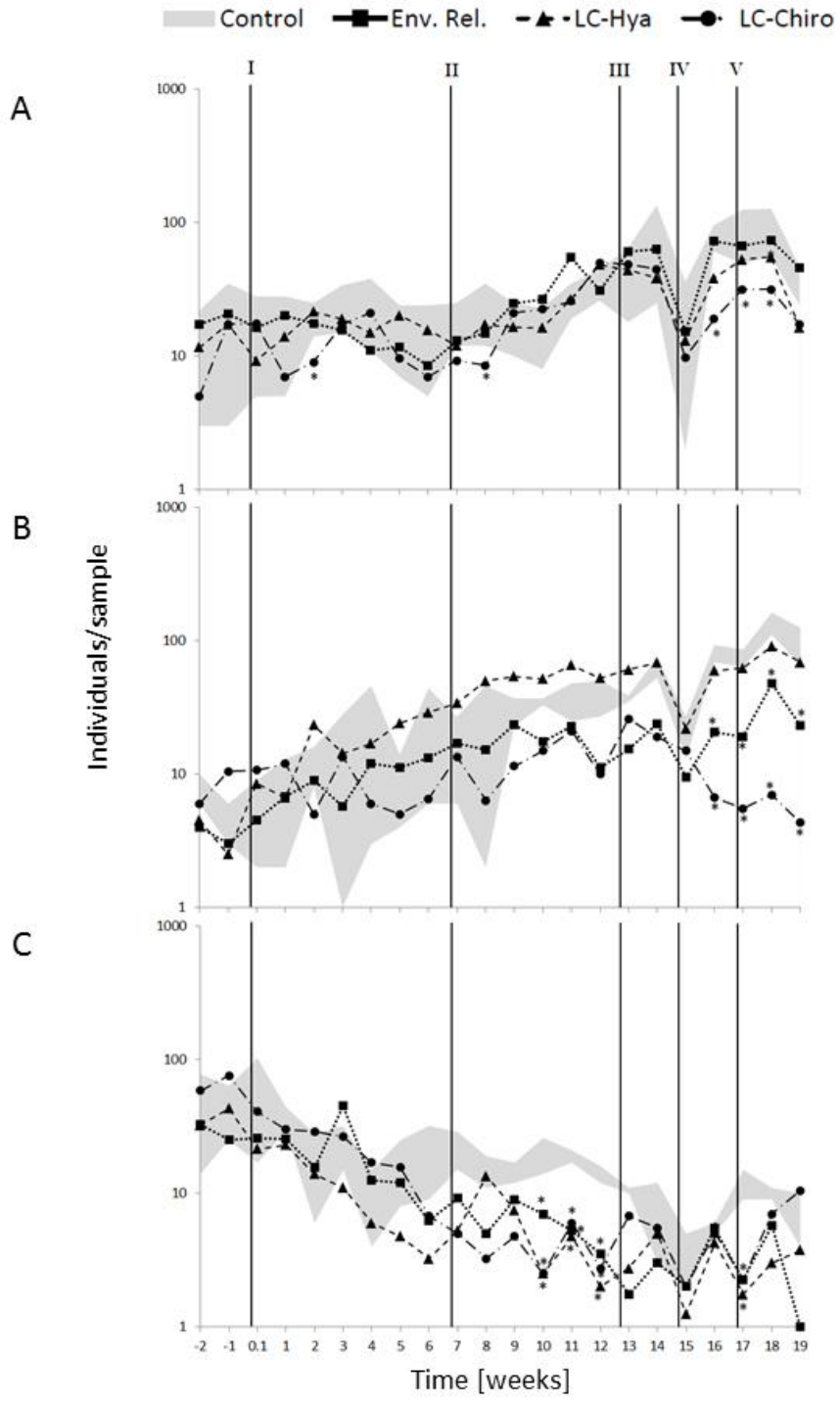


Figure 14 Change of abundance of Zygoptera (A), *Radix* sp. (B), and *Hyalella azteca* (C) for each treatment compared to the range of control values (grey area) over the sampling period. X-axis = time [weeks], Y-axis = individuals/sample. Asterisk represents a statistically significant difference of the treatment relative to the control ($\alpha = 0.05$). Env.Rel. = environmentally relevant concentrations, LC-Hya and LC-Chiro = lethal concentrations derived from previous laboratory assessments using *H. azteca* (LC-Hya) and *C. dilutus* (LC-Chiro). Vertical lines represent application events (at 0, 7, 13, 15, and 17 weeks past first application). Application concentrations for LC-Hya and LC-Chiro increased over time: I = LC10, II and III = LC25, IV and V = LC50

Power Analysis on taxa that displayed a significant effect in the RM-ANOVA, but not in the follow-up tests (Ephemeroptera, Diptera, Coleoptera, Planorbidae, *Culex* sp.), resulted in values between 0.91 and 0.99 supporting the statistical power of the data. While number of organisms was sufficient for conducting statistical analysis for Diptera, Planorbidae, and *Culex* sp., low numbers were observed for Coleoptera towards the second half of the study which might be a possible explanation for the missing significance in the follow-up tests. Power analysis on the abundance data of chironomidae (RM-ANOVA: P = 0.075, $F_{3,264}$ = 2.41, $\omega^2$ = 0.02) resulted in a power of 0.99 indicating a high number of sample sizes. PRC analysis of the macroinvertebrate community (containing abundances of all identified taxa) was not significantly affected by the pesticide treatment (P = 0.59).

Emergence of total abundance of flying taxa was not significantly affected by the pesticide treatment (RM-ANOVA, $F_{3,264}$ = 1.05, P = 0.821, Appendix A). However, Zygoptera showed a significant treatment effect in the RM-ANOVA (Table 9) that was represented by a decrease in emergence in week 11 for Env.Rel. and LC-Hya (Dunnett's test, P = 0.05, $F_{3,12}$ = 3.47, $\omega^2$ = 0.32). Power analysis on the emerging individuals of *Culex* sp., *Anopheles* sp., and total abundance resulted in a value of 0.99 indicating the statistical power of the data.

*Zooplankton response*

Eighteen zooplankton taxa were identified in the mesocosms. There were three cladoceran taxa, 11 rotifers, and two copepods. Nauplii and Ostracoda were not identified to a lower level due to taxonomic challenges. The rotifer *Trichocerca* sp. represented the largest portion of the macroinvertebrate community (22.3%), followed by the cladoceran *Chydorus* ssp. and total Ostracoda (19.3% each). PRC analysis of the zooplankton community data was significant (P = 0.002, $F_{16,352}$ = 14.2) with explanatory physicochemical parameter variables and time accounting for 24.5% of the community effect and the treatment effect accounting for 75.5% (Figure 15).

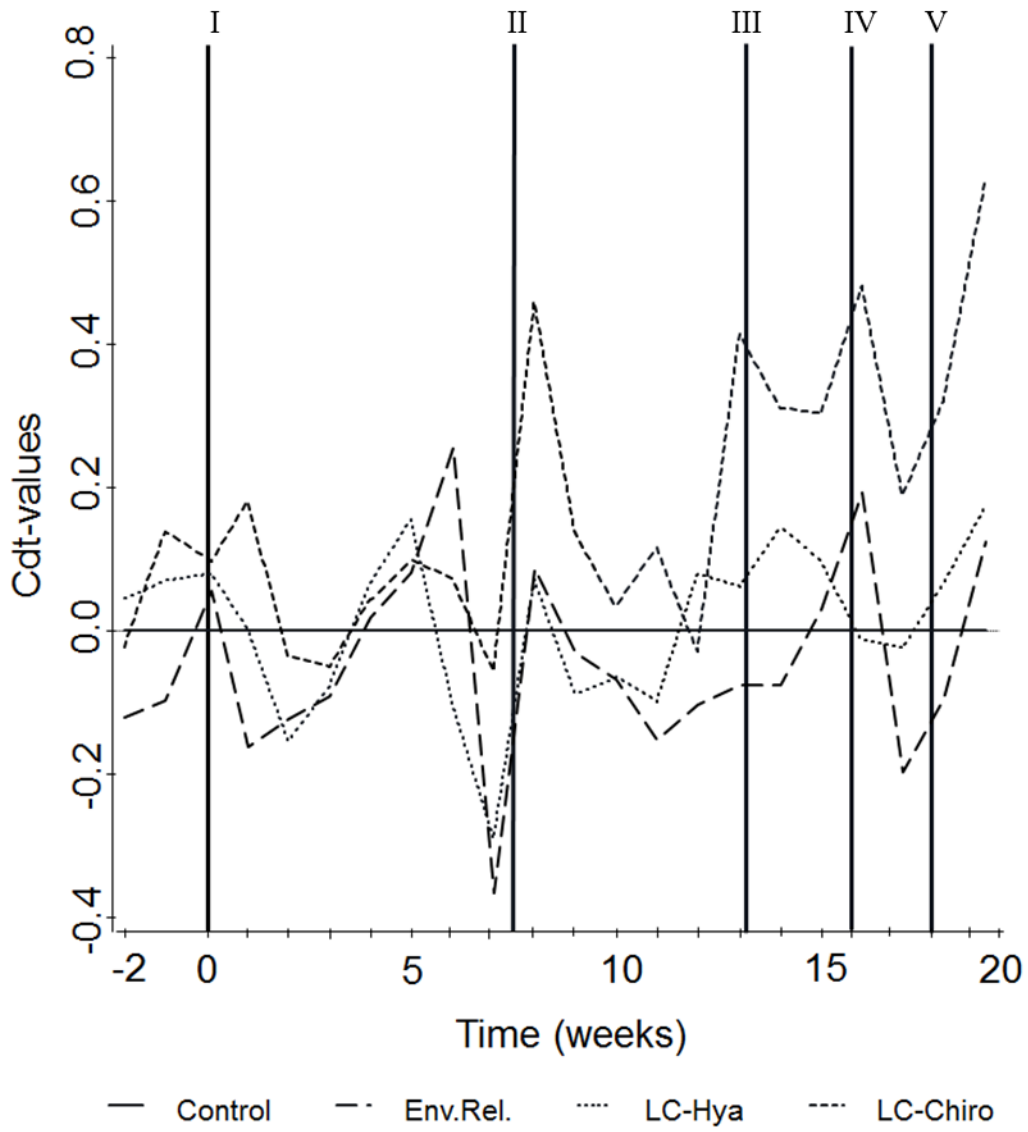


Figure 15 Principle Response Curves (PRC) indicating effects of applications of tertiary mixtures of permethrin, lambda-cyhalothrin, and chlorpyrifos on the zooplankton community. X-axis = time course (weeks) of the experiment, Y-axis = treatment effects (canonical coefficient = cd*t*), expressed as deviations from the control which is represented as an horizontal axis). Env.Rel. = environmentally relevant concentrations, LC-Hya, and LC-Chiro = lethal concentrations derived from previous laboratory assessments using *H. azteca* (LC-Hya) and *C. dilutus* (LC-Chiro). Vertical lines represent application events (at 0, 7, 13, 15, and 17 weeks past first application)

Greatest effects on the zooplankton community were observed in Env.Rel. following the first, second, and last application. These generated the greatest decrease below the control axis. Exposure to LC-Hya resulted in the strongest effect following the second application, but was not significantly different from the control on all other sampling days. Somewhat surprisingly, LC-Chiro represented the least severe effect on the overall zooplankton community. The overall community response showed a recovery (positive C*dt*-values) towards the end of the study for all treatments. All species scores for which the deviance was greater than |0.5| (and thus important for the community response)

displayed an opposite reaction compared to the PRC diagram (negative numbers, Table 10). Based on the species scores the most sensitive zooplankton species was *Daphnia magna* (-3.7), followed by Cyclopoidea sp. (-1.5), *Platyias patulus* (-0.9), *Trichocerca* ssp. (-0.9), Chydoridae (-0.8), and *Nauplia* ssp (-0.7).

Table 10 Species scores for zooplankton community. Taxa with a score greater than |0.5| (bold) indicate the affinity of a taxon to the Principle Response Curves (PRC) and are considered as important for the community reaction towards the treatment. Taxa with near zero weights either show no response or a response that is unrelated to the PRC

| Taxon | species score |
|---|---|
| *Anuraeopsis fissa* | 0.14 |
| *Ascomorpha* spec. | 0.31 |
| *Bosmina* ssp. | 0.15 |
| *Brachionus angularis* | 0.05 |
| *Brachionus calycifloris* | -0.02 |
| Calanoidae | 0.06 |
| **Chydoridae** | **-0.84** |
| **Cyclopidae** | **-1.53** |
| ***Daphnia magna*** | **-3.65** |
| *Euchlanis* spec. | 0.02 |
| *Keratella cochlearis* | 0.26 |
| *Keratella hiemalis* | 0.25 |
| *Mytilina mucronata* | -0.47 |
| **Nauplia** | **-0.71** |
| *Notholca* spec. | 0.36 |
| Ostracoda | 0.04 |
| ***Platyias patulus*** | **-0.89** |
| ***Trichocerca* spec.** | **-0.85** |

Ten out of sixteen zooplankton taxa were affected by pesticide mixtures. Results for each taxa are ordered by largest to smallest F-value gained from RM-ANOVAs (Table 11): Cyclopoidea, *Daphnia magna*, Copepoda, Cladocera, Ostracoda, Chydoridae, *Platyias patulus*, *Trichocerca* sp., Rotifers, and Brachionidae.

Table 11 Results of repeated measures ANOVA testing the effect of treatment on abundances of zooplankton taxa. Only taxa with significant P-values are listed herein.

| | d.f. | F | P | $\omega^2$ |
|---|---|---|---|---|
| Rotifers | | | | |
| Treatment | 3 | 3.09 | 0.033 | 0.01 |
| Error | 264 | | | |
| Brachionidae | | | | |
| Treatment | 3 | 3.03 | 0.036 | 0.02 |
| Error | 264 | | | |
| *Trichocerca* sp. | | | | |
| Treatment | 3 | 3.35 | 0.024 | 0.01 |
| Error | 264 | | | |
| *Platyias patulus* | | | | |
| Treatment | 3 | 5.07 | 0.003 | 0.04 |
| Error | 264 | | | |
| Ostracoda | | | | |
| Treatment | 3 | 6.39 | 0.001 | 0.03 |
| Error | 264 | | | |
| Cladocera | | | | |
| Treatment | 3 | 7.95 | < 0.001 | 0.04 |
| Error | 253 | | | |
| Chydoridae | | | | |
| Treatment | 3 | 5.45 | 0.002 | 0.03 |
| Error | 264 | | | |
| *Daphnia magna* | | | | |
| Treatment | 3 | 14.66 | < 0.001 | 0.10 |
| Error | 234 | | | |
| Copepoda | | | | |
| Treatment | 3 | 9.28 | < 0.001 | 0.06 |
| Error | 250 | | | |
| Cyclopidae | | | | |
| Treatment | 3 | 14.78 | < 0.001 | 0.09 |
| Error | 241 | | | |

d.f. = degrees of freedom, F = F-ratio, P = P-value. $\omega^2$ = Magnitude of effects.

LC-Chiro caused the greatest effects on individual zooplankton taxa, while Env.Rel. had the largest effect on the overall zooplankton community response. Significant RM-ANOVA results were investigated further using one-way ANOVA or Kruskal-Wallis test. The most sensitive taxon was Cyclopoidea that displayed a decrease in abundance in LC-Chiro in weeks 5 to 7 (P = 0.021 – 0.034, d.f. = 1, H = 4.50 – 5.33), 9 (P = 0.034, d.f. = 1, H = 4.50), 14 (P = 0.034, d.f. = 1, H = 4.50), and 17 to 19 (P = 0.020 - 0.049, d.f. = 1, H = 3.87 – 5.40), and in Env.Rel. in week 5 (P = 0.021, d.f. = 1, H = 5.33) and 18 (P = 0.027, $F_{1,4}$ = 11.52, $\omega^2$ = 0.64) (Figure 16A). Cyclopoidea represented 61.7% of the

Copepoda order (*Nauplia* sp.: 37.9%, Calanoidae: 0.30%), which is why the response of the order Copepoda was mainly driven be Cyclopoidea. Copepoda abundance decreased in week 5 in Env.Rel. and LC-Chiro (P = 0.021, d.f. = 1, H = 5.33), and in LC-Chiro in weeks 7 (P = 0.034, d.f. = 1, H = 4.50), 13 (P = 0.042, d.f. = 1, H = 4.13), and 17 – 19 (P = 0.020 – 0.038, d.f. = 1, H = 4.29 – 5.40).

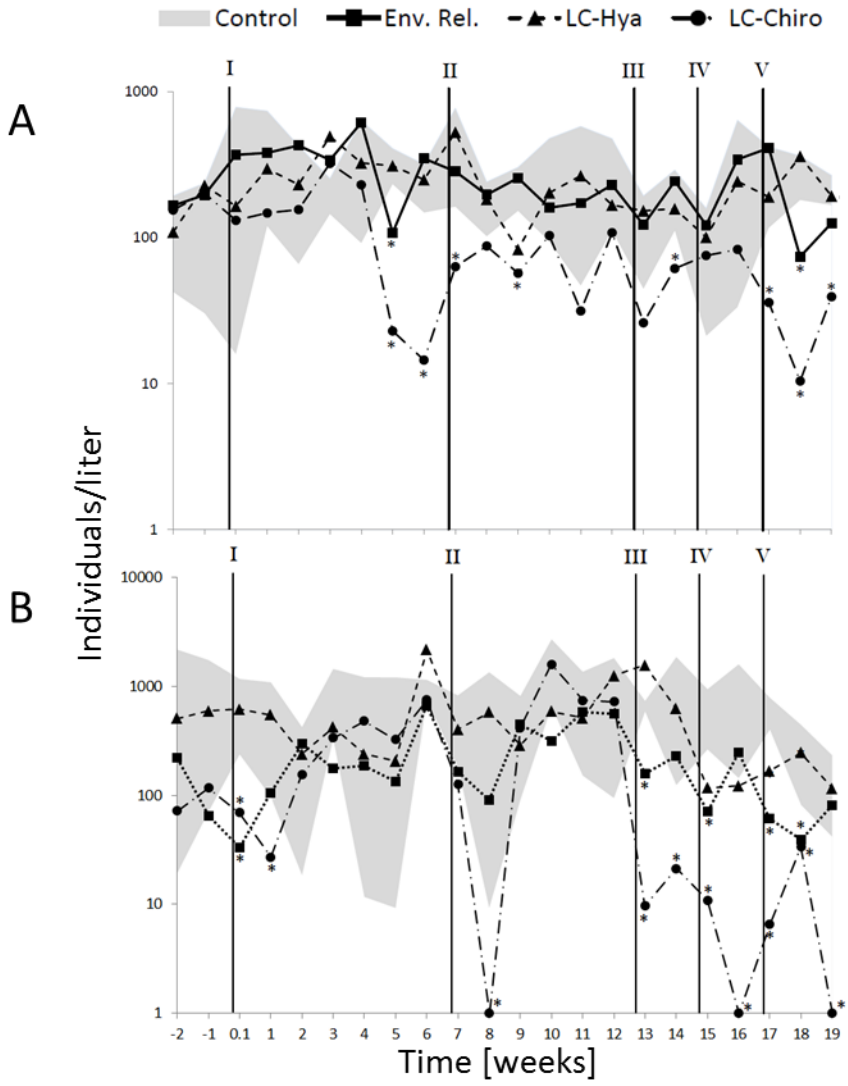


Figure 16 Change of abundances of cyclopoidea (A) and *Daphnia magna* (B) for each treatment compared to the range of control values (grey area) over the sampling period. X-axis = time [weeks], Y-axis = individuals/liter. Asterisk represents a statistically significant difference of the treatment relative to the control (α = 0.05). Env.Rel. = environmentally relevant concentrations, LC-Hya and LC-Chiro = lethal concentrations derived from previous laboratory assessments using *H. azteca* (LC-Hya) and *C. dilutus* (LC-Chiro). Vertical lines represent application events (at 0, 7, 13, 15, and 17 weeks past first application). Application concentrations for LC-Hya and LC-Chiro increased over time: I = LC10, II and III = LC25, IV and V = LC50

The second most sensitive zooplankton taxon was *Daphnia magna* with a decrease in abundance in Env.Rel. and LC-Chiro the day after the first application (P = 0.034, d.f. = 1, H = 4.50, and P = 0.030, d.f. =1, H = 4.50, respectively), in Env.Rel. in weeks 13, 15, 17 and 18 (P = 0.021 - 0.034, d.f.

= 1, H = 4.50 – 5.33), and in LC-Chiro in weeks 1 (P = 0.032, d.f. = 1, H = 4.50), 8 (P = 0.014, d.f. = 1, H = 6.05), and 13 to 19 (P = 0.014 - 0.028, d.f. = 1, H = 4.85 – 6.05) (Figure 16B). *D. magna* represented the most abundant taxa within the Cladocera order. In detail, Cladocera abundance decreased in week 13 (P = 0.049, $F_{3,11}$= 3.60, $\omega^2$ = 0.34), 16 (P = 0.036, $F_{3,12}$= 3.96, $\omega^2$ = 0.36), and 18 (P = 0.045, $F_{3,12}$= 3.64, $\omega^2$ = 0.33), while Chydoridae abundance decreased in LC-Chiro in week 15 (P = 0.03, d.f. = 1, H = 4.69) only.

Ostracoda abundance increased in LC-Chiro in week 7 (P = 0.05, $F_{3,12}$ = 3.48, $\omega^2$ = 0.32), but was negatively affected in LC-Hya in week 17 (P = 0.034, d.f. = 1, H = 4.50) and in week 19 (P = 0.021, d.f. = 1, H = 5.33).

Power Analysis on taxa that displayed a significant effect in the RM-ANOVA, but not in the follow-up tests (Brachionidae, *Trichocerca* sp., *Platyias patulus*), resulted in values between 0.95 and 0.98 supporting the statistical power of the data. Power analysis on the abundance data of taxa that did not result in significant RM-ANOVA (*Bosmina* sp., and *Nauplia* sp.) resulted in a value of 0.98 and 0.99, respectively, proving the statistical power of the collected abundance data. Numbers for *Ascomorpha* sp., *Brachionus* sp., and *Keratella* sp. were too low to use for statistical analyses.

### 4.5 Discussion

This study investigated both the mixture effects of three insecticides and analyzed pesticide fate in both the water column as well as the sediment, which allowed to compare biological responses to realistic concentrations for each sampling day over a period of four months, providing long-term information for pesticide risk assessment. Although pesticide mixtures and application rates varied both among treatments and over time in this study, some generalities can be drawn. The most sensitive species in this study were the two macroinvertebrate taxa *Radix* sp. and *H. azteca* which showed strong long-term effects, followed by the two zooplankton taxa *D. magna* and copepods. Effects on *D. magna* and *H. azteca* were acute (occurring within 24h of application). Copepods and *Radix* sp. responded after a lag time of one to five weeks following the first and second application, respectively. In contrast to these long-term responses, the later higher applications resulted in acute effects. These results are also consistent with other mesocosm and field studies that investigated the

effects of chlorpyrifos exposures applied singly; Cladocera and copepods were also among the most affected zooplankton taxa (Hua and Relyea 2014). The same was found in studies using pyrethroids in a review by Giddings *et al.* (2001), where the results of 7 mesocosm studies conducted in different systems and climates using cypermethrin and esfenvalerate revealed a consistent trend in sensitivity with amphipods, isopods, midges, mayflies, copepods, and cladocerans being most sensitive, and fish, snails, oligochaetes, and rotifers being the least sensitive.

A delayed decrease in copepod and *Radix* sp. abundances was observed in Env.Rel. and LC-Chiro on sampling days when concentrations were within the range of environmentally relevant concentrations reported in monitoring studies (Werner *et al.* 2010). For example, studies conducted on California surface waters detected lambda-cyhalothrin at concentrations of 1.4 to 27.0 ng/L, and permethrin between 4 to 470 ng/L in water samples (Weston *et al.* 2014). Chlorpyrifos was detected at concentrations between 1.2 to 226.0 ng/L (Weston and Lydy 2010). Pesticides were consistently detected in treated mesocosms, such that exposures were chronic. Chronic exposures to low concentrations of pyrethroids and organophosphates are common in field situations. Due to their neurotoxic mode of action, both chemical classes are likely to cause sublethal effects on invertebrates even at low concentrations (Desneux *et al.* 2007). Reproduction and swimming behavior are just two examples that could be affected by those compounds. Impaired swimming behavior of zooplankton species could for example affect their ability to obtain food, maintain their position in the water column, and avoid predators (Hanazato 2001). In combination with growth inhibition this has been shown to lead to failure to reach maturity and reproduce (Day and Kaushik 1987), and also suggests that a population exposed to pesticides could become even more sensitive to other abiotic and biotic factors depending on the duration of exposure.

Abundance of *H. azteca* was not significantly affected in the LC-Hya treatment that represented laboratory-based effect concentrations determined for *H. azteca*, however, *H. azteca* abundance was decreased following the fourth and fifth applications in LC-Chiro and at environmentally relevant concentrations (Env.Rel.). One reason for the lack of effect on *H. azteca* in LC-Hya, could be that the concentrations for permethrin and chlorpyrifos were lower in Env.Rel. compared to LC-Hya and LC-

Chiro, but the amount of lambda-cyhalothrin was greater in Env.Rel. and LC-Chiro; this indicates that lambda-cyhalothrin could be the most potent pesticide out of the three.

Another reason why *H. azteca* may have responded differently in the mesocosm study compared to the laboratory exposure is the different chemical behavior of pyrethroids in ambient water. In this study, pyrethroids rapidly dissipated from the water column, while chlorpyrifos was still detectable, even after six weeks of exposure. Pyrethroids rapidly dissipate from the water phase and adsorb to particulate matter as they are highly hydrophobic compounds of low water solubility and high $K_{OW}$ values ($K_{OW} > 6$ for pyrethroids, versus $K_{OW}$ for chlorpyrifos = 4.7) resulting in a high affinity to any type of surface (Laskowski 2002), including mineral and organic particles (e.g., algal and leaf material) in both suspension and in sediments (Maul *et al.* 2008). This level of partitioning also explains why sediment concentrations of pyrethroids did not increase significantly, even though pyrethroids were not detected in the water column.

While copepods and *D. magna* are filter feeders, *H. azteca* are epibenthic grazers and gill-breathers that primarily occur at the interface of the water column and sediment or detritus. As a shredder species, *H. azteca* rely on leaf material and detritus as a food source (Maul *et al.* 2008). The binding properties of pyrethroids have been shown to inhibit their detrital degradation (Lee *et al.* 2004), suggesting an accumulation of these compounds on particulate matter. Although unavailable for cuticular uptake, particle-bound compounds may be bioavailable via dietary exposure. Pyrethroid-spiked sediments and particles showed negative effects on invertebrate drift (Schulz and Liess 2001) suggesting that particle-bound pyrethroids represent an additional route of exposure to detritivores or filter feeding organisms. This could lead to a similar dietary exposure of the three taxa. Effects of dietary exposure of pyrethroids has been reported to cause sublethal effects such as abnormal swimming behavior and reproduction (Werner *et al.* 2002) confirming that dietary exposure is an important route of exposure. Further, the gills of *H. azteca* represent another potential uptake route for this species due to their large surface area further explaining their significant response to the pesticide exposure. This kind of mechanism was confirmed in a study using another crustacean species, *Penaeus monodon* (black tiger shrimp), that found that the pyrethroid deltamethrin strongly affects oxidative stress biomarkers in the gills (Huynh Thi *et al.* 2012).

Odonates were the largest predators in the mesocosm. Studies suggest that impact from pesticides may be further magnified in the presence of predators cues (Campero *et al.* 2007), thus predators represent an additional natural stressor that is especially important for prey population dynamics in aquatic ecosystems (Preisser *et al.* 2005). For example, pesticide exposure combined with predator cues (mayfly larvae and damselfly larvae) caused synergistic effects on survival and biomass of water bugs (Corixidae) (Trekels *et al.* 2013). Due to the dominant presence of odonates in this study, abundance of chironomid larvae could have been decreased across all tanks (controls as well as treatments) as odonates are known to feed heavily on chironomids (Saha *et al.* 2014). Odonates showed a treatment effect towards the end of the study, possibly caused by the significant decrease of their prey items such as *D. magna* and *H. azteca*. Perhaps this response was not long-term because they are generalist predators and other prey was available (i.e., *Culex* sp. or *Anopheles* sp.). However, it is important to note that Zygoptera was the only insect for which a decrease in emergence was detected. It has been shown that insecticides potentially impact insect emergence. For example, in a study using the neurotoxic carbamate insecticide decreased the emergence success of the damselfly *Xathocnemis zealandica* and increased the fluctuating asymmetry in wing cell patterns in both laboratory and mesocosm experiments (Hardersen *et al.* 1999).

The decrease of the *Radix* sp. snail that started in week 5 in all three treatments may have been caused by a direct effect by the pesticide exposure in combination with an indirect effect due to predation pressure. In a study using the pyrethroids cypermethrin and alphamethrin the oxidative metabolism in the hepatopancreas and the ovotestis tissues of the freshwater snail, *Lymnaea acuminate*, were altered, causing increased numbers of eggs, but reducing the survival rate of the snails 28 days after hatching *(Tripathi and Singh 2004)*. *Radix* sp. belongs to the same family Lymnaediae as *Lymnaea acuminate* which could be an indicator for a similar response of *Radix* sp. and could thus explain the delayed negative response of the snail in the present study. Abundance may also have been indirectly affected by the dominance of Anisoptera in the system. Dragonfly nymphs are known to feed on aquatic snails among other invertebrate species, and with other invertebrates such as *H. azteca* and *D. magna* declining in the system due to the pesticide exposure, their feeding patterns may have shifted towards the snail.

Abundances of *H. azteca* and *Radix* sp. populations were decreased for several weeks and did not recover as quickly compared to other macroinvertebrate species. Recovery of affected populations following pesticide exposure is governed by many factors such as the persistence and type of the compound, time of year when the exposure occurs, distance to unexposed habitat with recolonization sources, and species traits related to life history and dispersal (Moe *et al.* 2013). Due to their rapid growth rates and short generation times, most aquatic invertebrates are capable of rapid population growth allowing a quick recovery following pesticide exposure (Giddings *et al.* 2001). Recovery can also be supported by areas with low pyrethroid concentrations that can serve as refuges and sources for population recolonization. Under natural conditions, immigration from nearby unexposed areas can lead to quick recovery of affected populations. This is especially true for species with an adult winged stage, such as species of the order Diptera, for which external recolonization in the form of successful deposition of eggs by winged adult females as well as their capability to escape from the pesticide-stressed ecosystems due to emergence can contribute to their recovery (Brock *et al.* 2009). However, in enclosed mesocosm experiments this is unlikely for fully aquatic species, such as *Radix* sp. and *H. azteca.* This was confirmed in a study investigating the effects of pyrethroids using 25$m^3$ pond mesocosms, where recovery was minimized for the large Crustacea *Gammarus* and *Asellus* due to the enclosed nature of the system (Farmer *et al.* 1995).

Although application concentrations for Env.Rel. were kept constant for each application and pesticide concentrations in the water or sediment did not accumulate, the greatest effects were observed towards the end of the study (≥ week 16). A possible explanation for this could be the decreased ambient temperature towards the end of the season. Lower temperatures typically reduce lethal effects of pesticides such as organophosphates, but cause the opposite pattern for pyrethroids. In a study using permethrin and bifenthrin, toxicity to *H. azteca* decreased by a factor of 1.9 – 2.3 per 5°C increase in temperature, while chlorpyrifos toxicity was nearly temperature independent (Weston *et al.* 2009). Change in temperature can also affect metabolic function and toxicokinetic rates in organisms. Harwood *et al.* (2009) noted that the decrease in chlorpyrifos toxicity combined with temperature increase caused decreased biotransformation ability in *C. dilutus.* Similar inhibiting effects on biotransformation were also reported for pyrethroid toxicity at lower temperatures

(Narahashi 2002). The combination with factors such temperature-induced effects (Harwood *et al.* 2009) and interspecific interactions such as predation (Preisser *et al.* 2005) or competition (Campero *et al.* 2007) could lead to a magnified impact on those populations and thus the entire food web (Geist 2011). These results indicate that pesticide-induced changes in food web dynamics may be strongly underestimated in laboratory-based risk assessment. While laboratory-based toxicity tests performed under standardized conditions are a valuable tool for risk assessment, the long-term effects on aquatic communities may not be accurately determined, which is also an increasing concern in the light of global climate change (Moe *et al.* 2013).

This study confirmed the major advantage of using model ecosystems such as mesocosms that simulate realistic exposures of interactive trophic levels of aquatic organisms to pesticides, in that effects were not accurately predictable from laboratory studies. Both direct and indirect effects on a wide array of species were assessed while allowing for realistic interactions between the various populations within a community and more complex population responses to insecticides. Moreover, from a chemical fate perspective, dissipation and accumulation of a chemical dose was observed under realistic conditions. Mesocosms can help to develop generalizations about how these insecticides can alter aquatic ecosystems. Further, focused laboratory assessments at realistic concentrations that have highlighted toxic sublethal effects are crucial for interpreting the community response data and allow making more realistic assumptions about the effects in the field.

## 4.6 Conclusion

In this study, we demonstrate that the direct and indirect effects of the insecticide mixtures on macroinvertebrates and zooplankton had unique effects on biological and abiotic variables. Additionally, though most insecticides are meant to act rapidly and then degrade, they can continue to impact natural systems over a longer period of time, even when bound to particles. Finally, aquatic systems commonly face complex mixtures of insecticides that can, compared to insecticides applied individually, have unanticipated positive and negative direct consequences that can lead to indirect effects at the community level and alter abiotic variables. These results highlight the importance of integrating multiple organizational levels and long-term exposures in ecological risk assessments of

insecticides. Taking into account the response and recovery of species to insecticides in complex community scenarios can facilitate our ability to make predictive and mechanistic generalizations about the role of insecticides in shaping patterns of species abundance in natural systems.

# 5. Overall Discussion

This thesis investigated the effects of two classes of insecticides, pyrethroids and organophosphates, applied individually and in mixtures, on lethal and sublethal responses of invertebrates in laboratory toxicity exposures (chapters 2 and 3). Further, the results from the laboratory exposures in addition to environmentally relevant concentrations were applied to outdoor mesocosm exposures to analyze effects on community structure and pesticide fate (chapter 4). This approach allowed for a comparison of mixture toxicity assessments across different biological levels, endpoints, and test duration, each representing a different level of complexity (Figure 17).

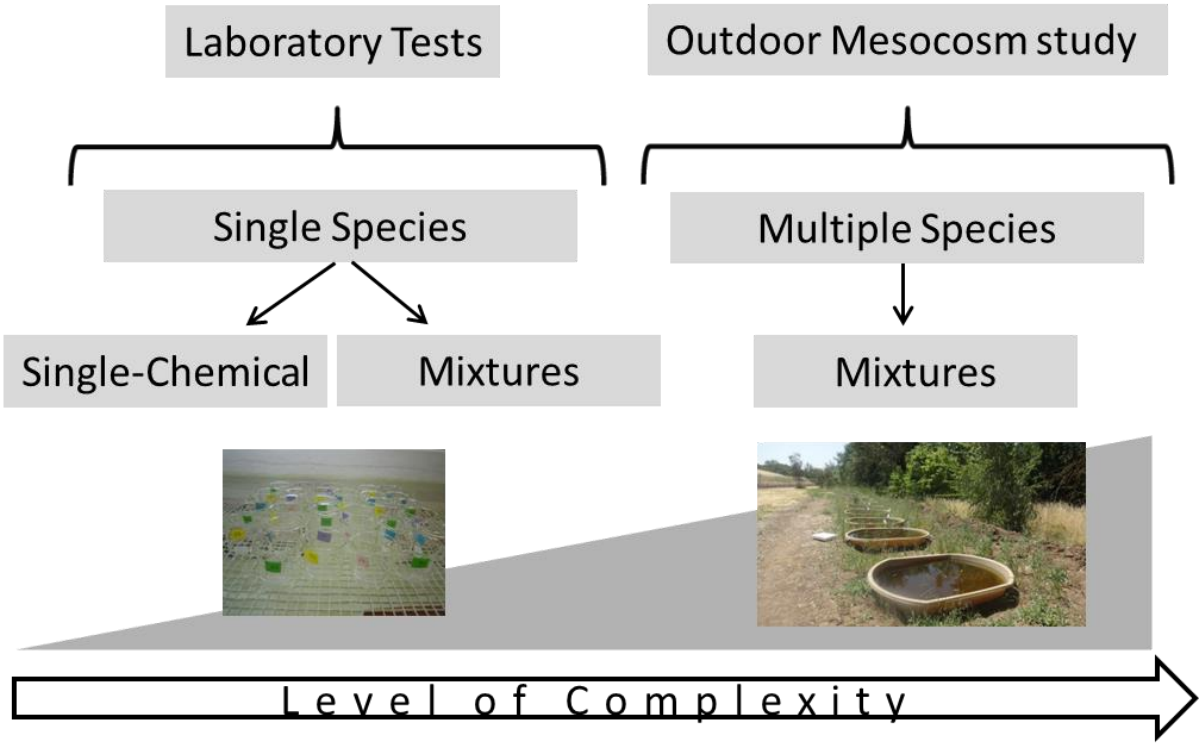


Figure 17 Level of complexity in single species exposures compared to mesocosm studies

In the 10-day laboratory assessments in chapters 2 and 3, sublethal effects on growth and motility were detected at 1/8 of an LC50, at concentrations that were often below the limit of detection of chemical analysis and below concentrations that are typically detected in the environment. The effects of mixture exposures, at relative toxic concentrations, were less severe than those observed in the single exposures indicating an antagonistic response (chapter 3). In the mesocosms, the effects of a series of applications of tertiary contaminant mixtures resulted in negative effects on the abundance of

multiple species, causing significant long-term decrease of *H. azteca* and *Radix sp.* population, and zooplankton species such as copepods and *D. magna* at both the laboratory-based treatment (LC-Chiro) and environmentally relevant concentrations (chapter 4). The environmentally relevant concentrations had the greatest effect on the zooplankton community than the laboratory-based treatments. This indicates that lethal concentrations determined in laboratory single-species tests may not necessarily reflect the effects observed in the environment as the ratio as well as the concentrations of each contaminant in a mixture may affect the joint toxicity.

In this project, laboratory as well as mesocosm assessments provided essential information for understanding mixture toxicity and evaluating pesticide mixture effects on aquatic ecosystems. The results highlight the importance of integrating multiple organizational levels and long-term exposures in ecological risk assessment to facilitate the ability to make predictive and mechanistic generalizations about the role of insecticides in shaping patterns of species abundance in natural systems. The advantages and disadvantages of the different test designs will be discussed in detail in the following.

### 5.1 Different Scales of Investigation – From Single Species Tests to Mesocosm

Traditionally, ecotoxicologists have relied on short-term, single-species laboratory tests to assess the environmental risk of contaminant mixtures on aquatic biota (Faust *et al.* 2000). This approach can be informative in understanding the direct consequences of contaminants on individual species, under controlled conditions, as laboratory single-species toxicity tests are an efficient and economical way to assess pesticide effects in a small space which many laboratories can afford (Stanley *et al.* 2005). In addition, laboratory exposures allow the use of a large number of replicates and different treatment concentrations and have the potential to be easily modified (e.g., exposure temperature or water quality parameters) as well as to study specific mechanistic endpoints for a species of interest (e.g., Connon *et al.* 2008; Weston *et al.* 2009). Laboratory single-species approaches used in chapter 2 and 3 allowed for the investigation of sublethal effects of pesticides, applied individually and in mixtures, on growth and motility within 10-day exposures. Knowledge gained from such single

species studies is crucial for guiding field-based studies towards predicting and interpreting overall environmental effects on aquatic communities; as discussed in chapter 4. Generally, single-species tests do not represent a level of environmental realism that allows a practical approximation of exposure, and can thus lead to limited or misleading conclusions about the community effects of contaminants.

There are a number of advantages of mesocosm studies which cannot be reproduced easily and accurately under short-term laboratory conditions (Chappie and Burton 1997). First, diversity and species complementarity can increase over longer time scales because of a greater temporal variation in conditions (Cardinale *et al.* 2007), which facilitates the expression of differences among species in response to seasonal changes (Stachowicz *et al.* 2002) and allows for sufficient interactions among species and thus the expression of potential chronic sublethal or indirect effects as well as recovery of the population (Cardinale *et al.* 2007; Tilman *et al.* 2013). Additionally, seasonal changes such as variation in light, dissolved oxygen, and temperature, but also organic matter and suspended solids, are known to affect the bioavailability of some contaminant classes. This in turn can impact organismal physiology, potentially altering susceptibility to both anthropogenic and natural stressors (Bervoets *et al.* 1996; Bereswill *et al.* 2013).

To perform a thorough risk assessment and gain a clear understanding of exposure effects, a quick and cost-effective approach is often inevitable. As such, short-term laboratory tests represent ideal high throughput assessments. Toxicity testing in surrogate systems, such as mesocosms, should not be considered as a replacement for laboratory tests, but is highly suitable for evaluating specific scenarios (e.g., contaminant mixtures) that have been identified as problematic through small scale approaches. Both approaches should be used together in a complementary manner to develop a weight-of-evidence approach for contaminants of concern.

### 5.2 Test Endpoints - From Sublethal Responses to Community Response

As effects of contaminants can impact and manifest at different levels of biological organization, from the molecular level (i.e., changes in the gene transcription and expression patterns) to effects on

entire communities (Geist 2011), measuring a suite of indicators is often necessary to assess ecological integrity in ecotoxicological testing (Adams *et al.* 1992; Karr 1993) (Figure 18).

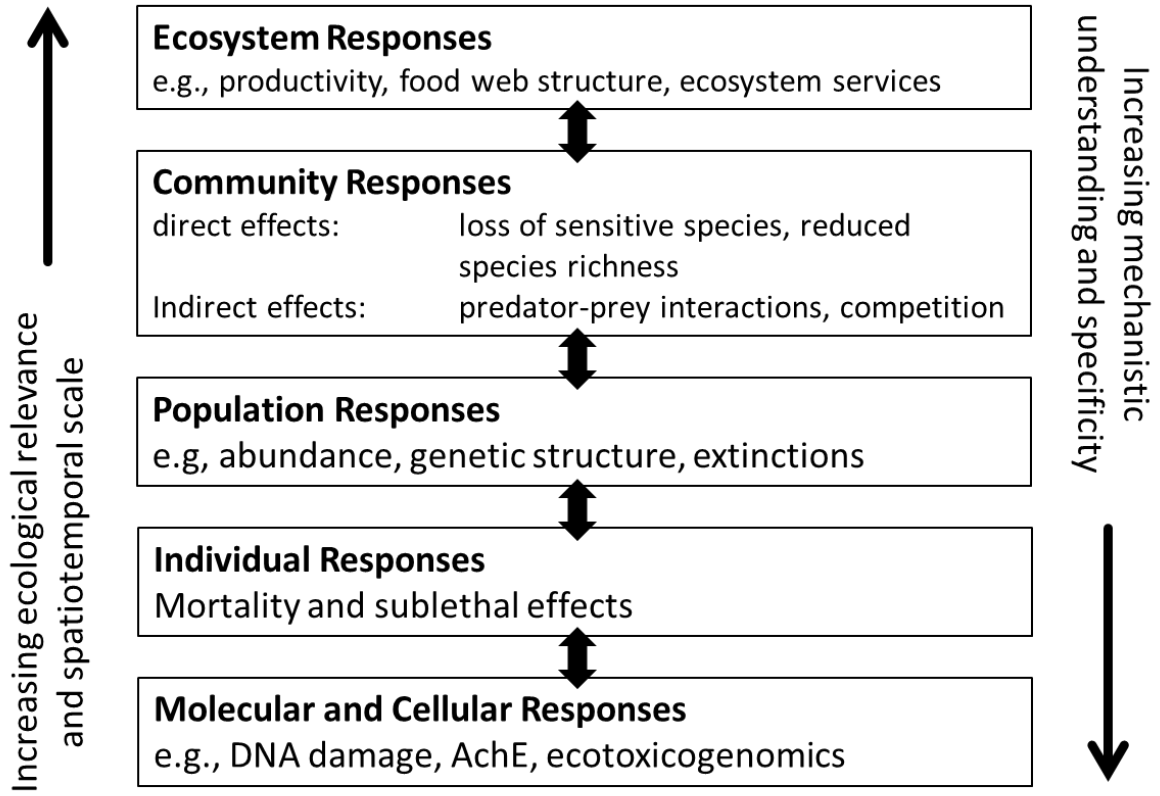


Figure 18 Responses caused by exposure to contaminants across different levels of biological organization, considering increasing ecological relevance and spatiotemporal scale as well as mechanistic understanding and specificity. Modified from Geist (2011) and Clements (2000)

Following contaminant exposure, responses at lower levels of biological organization (molecular and cellular responses) occur more rapidly and are generally stressor specific. Additionally, they provide early signs of toxicological effects on populations and are generally better understood in terms of mechanisms of action (Clements 2000). Responses at higher levels (communities and ecosystems) occur at broader spatiotemporal scales and provide a direct linkage to ecological effects. Hence they have greater ecological relevance (Cairns 1983), but often lack mechanistic understanding of cause and effect.

Laboratory assessments support the understanding of direct consequences of contaminant exposures on individual species, such as their mortality, but even more importantly their sublethal response to exposure; e.g., reproduction (Tripathi and Singh 2004; Mahar and Watzin 2005), growth

(Barata *et al.* 2012; Hasenbein *et al.* 2015), and gene response (Geist *et al.* 2007; Connon *et al.* 2009). Sublethal endpoints represent sensitive and ecologically significant approaches to understand sensitive organism level responses to chemical stressors. As sublethal effects may magnify and manifest in the population and thus in the food-web, such endpoints provide population relevant information for ecological risk assessment. Especially in waters where contaminant concentrations are detected below levels that result in direct mortality, sublethal endpoints are of increasing importance (Scholz *et al.* 2012). Additionally, many contaminants may cause long-term health impacts or reproductive impairment, which are often not detectable using traditional toxicity testing methods (e.g., Christensen *et al.* 2005; Brander *et al.* 2012; Connon *et al.* 2012). In chapters 2 and 3, it was demonstrated how the exposure to pyrethroids and organophosphates can result in negative effects on invertebrate motility and growth within a 10-day exposure, at concentrations in the range typically detected in the environment, or even in some cases, below the limit of detection of current-use analytical methods. As neurotoxic compounds pyrethroids and organophosphates are known to cause sublethal effects such as impaired swimming behavior, which can reduce feeding ability, predator avoidance, and impact reproduction (Little and Finger 1990; Floyd *et al.* 2008; Holomuzki *et al.* 2010). Motility is a useful and ecologically important endpoint for the detection of low-level pesticide effects on invertebrates. This was also confirmed in other studies that used invertebrates, where swimming behavior represented the most sensitive endpoint to use for risk assessment of neurotoxic compounds (Rubach *et al.* 2011; Chen *et al.* 2014). When growth is considered from the perspective of its functional processes within ecosystems, a reduction in growth results in an overall reduced biomass, or productivity, and therefore a decrease in the quantity of food available to higher trophic levels (Sibley *et al.* 1997). Further, changes in growth are likely to be strongly related to effects on reproductive processes (Tolba and Holdich 1981; Moore and Dillon 1993). Similar effects have been observed by other researchers, where effect-based endpoints, designed to assess sublethal impairments, were useful predictors of deleterious effects associated with contaminated water and sediments (Maul *et al.* 2008; Connon *et al.* 2012; Deanovic *et al.* 2013; Rasmussen *et al.* 2013). For example, the application of a microarray on *D. magna* determined impacts of contaminants that were directly linked to somatic growth and development (Connon *et al.* 2008). These findings indicate that

the integration of sublethal endpoints in ambient water monitoring and pesticide regulation efforts could improve identification of low-level pesticide concentrations and help to further understand negative effects on food webs and community structure in aquatic environments.

While assessing lethal and sublethal endpoints in laboratory exposures it is crucial to understand the mechanistic and chronic consequences of contaminants on individual species; as contaminants can also have indirect, and potentially cascading effects at the population and community levels (Fleeger *et al.* 2003; Relyea and Hoverman 2006; Peters *et al.* 2013). Such ecological alterations can be caused by impaired (sublethal effects) or decimated (lethal effects) species that may initiate a trophic cascade (indirect effects mediated through consumer-resource interactions) or a release from competition that secondarily leads to responses in tolerant species (Fleeger *et al.* 2003). For example, crustacean zooplankton populations represent an important component of freshwater ecosystems, as effective grazers on most planktonic micro particles and periphyton. Thus, direct toxic effects on the crustacean zooplankton may result in a reduced grazer control of phytoplankton, protozoans, and rotifers, which may lead to cascading indirect effects on resistant species in other trophic levels and thus an altered biomass and species composition of the communities (Friberg-Jensen *et al.* 2003).

Other factors that are important to be considered in environmental risk assessment, and that can be monitored using mesocosms, are the response and recovery of community and ecosystem properties following chemical disturbances under more natural conditions (Relyea and Hoverman 2006). The capacity of an aquatic ecosystem or community to recover after contaminant perturbation depends on factors such as the persistence and bioavailability of the toxicant, the life-history attributes of organisms, and the proximity and location of re-colonization sources (Fairchild *et al.* 1994). For example, zooplankton populations generally recover more rapidly due to their *"r-"* selected reproductive strategy and short generation time (Pianka 1970). In contrast, other aquatic invertebrate and fish populations exhibit longer recovery times due to increased generation times as well as frequently restricted (seasonal, spatial, or physically obstructed) re-colonization potential. However, the recovery of fish populations and fully aquatic invertebrates (vs. insects) cannot be realistically determined with mesocosm studies due to the lack of re-colonization sources. Moreover, most insecticides are designed to act immediately and degrade quickly (Newman, 1992), but short-term

consequences can potentially lead to unanticipated lethal or sublethal effects on communities that may last long after the insecticide has degraded (i.e. lag effects) as was observed in chapter 4.

### 5.3 Evaluation and Prediction of Mixture Toxicity Effects

Aquatic organisms are exposed to complex mixtures of contaminants, which originate from many point and nonpoint sources, throughout their life cycle. In combination with other stressors, such as climate change, habitat degradation or introduced species, they are exposed to potentially increasing stress situations (Dudgeon *et al.* 2006). Thus, the evaluation of potential hazards of chemicals, and especially that of chemical mixtures, represents one of the greater challenges in ecotoxicological research, environmental risk assessment, and regulatory toxicology.

Concentration Addition (CA) and Independent Action (IA) are two traditional models that have been widely utilized for the calculation of mixture toxicity predictions and mixture toxicity assessments in pesticide regulation (e.g., Altenburger *et al.* 2003; Jonker *et al.* 2004; Syberg *et al.* 2009). The choice of model is generally based on the mode of action (CA: similar MoA; IA: different MoA) (Wang *et al.* 2015), but scientific findings over the last decades indicate that CA can be applied regardless of mode of action and thus should be used as a general mixture prediction model (Syberg *et al.* 2009). Additionally, toxicological interactions; additivity, synergisms or antagonisms, and their respective effects can occur independently of mode of action (Cleuvers 2003; Lydy *et al.* 2004; Belden *et al.* 2007). For example, Cedergreen *et al.* (2012) compared synergism in binary and tertiary mixtures and found that binary mixtures displayed the strongest degree of synergy (deviation from the CA model). Similar observations have also been reported in numerous other studies (see: Woods *et al.* 2002; Cooper *et al.* 2009; Rosal *et al.* 2010; Wang *et al.* 2011). Thus, Cedergreen *et al.* (2012) concluded that knowledge of the strongest degree of synergy of a binary mixture can be used to determine the size of safety factors used for complex mixtures including potential synergists, as suggested by Thompson (1996). Further, interactions of chemicals do not occur uniformly or predictably and are known to be concentration dependent (Crofton *et al.* 2005; Rodney *et al.* 2013). For example, Laetz *et al.* (2008) found that the interaction of mixtures of organophosphate and carbamate pesticides on brain acetylcholinesterase activity in coho salmon was best predicted by

additivity at low doses and by synergy at high doses. Interaction in a mixture may also be concentration ratio dependent, but only few models have efficiently dealt with the influence of magnitudes of exposure and dependence on the ratios of the component chemicals (Rodney *et al.* 2013). Given this number of uncertainties in predicting mixture effects, it has been proposed that safety factors should be increased for environmental risk assessment, if compounds that are known to cause synergistic effects are present in a mixture (Thompson 1996). Nevertheless, it is unknown if, and how, the maximal level of potentiation can be predicted in order to assess the needed increase for such specific safety factors (Cedergreen *et al.* 2012).

Other researchers have attempted to establish a more reliable approach to predict mixture toxicity. For example, Chou (2006) developed the combination index (CI) – isobologram equation that is widely used for drug interaction evaluations in pharmacology, and allows quantitative determinations of chemical interactions at different concentrations as well as effect levels. Rodea-Palomares *et al.* (2010) applied this method for ecotoxicological risk assessment, which was subsequently used to investigate the interactions among chemicals (e.g., Rosal *et al.* 2010; Boltes *et al.* 2012; Rodea-Palomares *et al.* 2012). Although the CI analyses provide information on the nature and extent of chemical interaction, this method does not include concentrations-effect curves and the corresponding contaminant concentrations either as single chemical or combinations (Zhao *et al.* 2004). Other models have been proposed to evaluate interacting mixtures, but most are descriptive in nature; simply testing the significance of deviation from additivity (e.g., the empirical model MIXTOX), and require all ratios and dose or concentration levels to be equitoxic (Charles *et al.* 2002; Jonker *et al.* 2005).

Some studies that tested the potential for pesticide interactions at environmentally relevant concentrations or ratios of contaminants, did not necessarily detect synergistic effects (Teather *et al.* 2005; Junghans *et al.* 2006; Brander *et al.* 2009). For example, George *et al.* (2003) evaluated the effects of pesticide mixtures on zooplankton abundance in outdoor microcosms based on North American surface water monitoring data. Effects on abundance were additive for the binary mixtures (CA) while results of the tertiary mixture were consistent with an independent action (IA) response. Based on these findings, the CA model may be a conservative choice for estimating effects on aquatic

organisms when pesticides are considered at environmentally relevant concentrations. This also indicates that more studies are needed where environmentally relevant concentrations and ratios are integrated, in order to allow for more dependable predictions of mixture toxicity effects in aquatic environments. Further, comparing laboratory ecotoxicological data to field studies is critical to make well-informed regulatory decisions for the use of pesticides in the environment. However, without reference points closer to the real world, judging the relevance of bioassay results or the reliability of inferences drawn from them is difficult. To assess the complex effects caused by pesticide mixtures, sophisticated approaches are needed to evaluate the direct and indirect consequences of insecticides across multiple levels of biological organizations, in order to develop generalizations about how these chemicals can alter aquatic systems.

# 6. Final Conclusion and Outlook

This thesis compared different approaches for assessing toxic effects of pesticides at various biological endpoints and levels of complexity, by combining laboratory and mesocosm exposures using invertebrate species as primary targets. By using laboratory-based toxicity tests, it was possible to determine ecologically relevant sublethal effects such as changes in growth and motility under controlled conditions within short periods of time. Mesocosms on the other hand, allowed to evaluate long-term community and food-web effects, and as such represented a much more realistic exposure scenario. An integrated assessment based on the combined consideration of both approaches provided essential information for understanding mixture toxicity and evaluating their effects on aquatic ecosystems, which can be applied in risk-assessments of contaminants of concern.

As discussed in chapters 2 and 3, the integration of ecologically relevant sublethal endpoints, such as growth and motility, must be considered in future risk assessment and monitoring efforts of pesticides, as they can help to avoid either under- or overestimation of toxicity, especially at low concentrations that are below the limit of detection of current-use analytical methods. If a negative response at the species-level has been detected, long-term multi-species studies are needed to monitor effects at the community level. As discussed in chapter 4, integrating multiple organizational levels and long-term exposures, which investigate the response and recovery of species in ecological risk assessments of pesticides, can facilitate the ability to make predictive and mechanistic generalizations about the role of insecticides in shaping patterns of species abundance in natural systems. It was highlighted in chapter 4 that mesocosms represent an increased complexity from bench-top laboratory tests and can be a useful approach for the ecological effect assessment of pesticide mixtures since they closely simulate natural conditions. However, they lack standardization in terms of biological communities, which is why a greater degree of standardization is desired in the future as to allow a wide application of this approach in regulatory assessments. Further, the mesocosm system used in this thesis does not fully represent the complexities of natural environment, for example due to the absence of fishes and other predators that are present in many natural ecosystems. Especially fish

larvae and juveniles are considered to be highly vulnerable life stages (Holdway *et al.* 1994), and highly sensitive to pesticide exposures. Additionally, in many agricultural and urban areas, the periods of peak pesticide application coincide with the spawning season of multiple fish species (Moyle 1976). Ideally, future studies would compare the bottom-up approach as demonstrated in this study with a top-down effect evaluation considering more complex food webs that also include fishes. It is evident from the results of this thesis that insecticides, both in mixtures as well as individually, represent a major threat to aquatic organisms, causing negative effects determined using sublethal endpoints on individual organisms as well as on populations and communities even at environmentally relevant concentrations. However, these results should be compared with other classes of contaminants as well as different mixture ratios thereof, such as herbicides or fungicides that have the potential to impact other components of the food-web, which in combination could lead to severe cascade-effects within the community. In the light of climate change and the associated increase of pests, the use of pesticides is predicted to increase over the next decades. Therefore, future studies that integrate sublethal endpoints in combination with focused and standardized mesocosm studies are crucial to understand the impact of contaminant mixtures on aquatic communities.

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her deep love and strength will always
be in my heart.

# 8. Publication List

## 8.1 Peer-reviewed Publications Included in this Thesis

Hasenbein S, Connon RE, Lawler SP, Geist J (2015): A comparison of the sublethal and lethal toxicity of four pesticides in *Hyalella azteca* and *Chironomus dilutus.* Environmental Science and Pollution. DOI: 10.1007/s11356-015-4374-1

Hasenbein S, Lawler SP, Geist J, Connon RE (2015): The use of growth and behavioral endpoints to assess the effects of pesticide mixtures upon aquatic organisms. Ecotoxicology. DOI: 10.1007/s10646-015-1420-1

Hasenbein S, Lawler SP, Geist J, Connon RE: A long-term assessment of pesticide mixture effects on aquatic invertebrate communities. Environmental Toxicology and Chemistry. *In Press.*

## 8.2 Peer-reviewed Publications not Included in this Thesis

Jeffries KM**,** Komoroske LM, Truong J, Werner I, Hasenbein S, Hasenbein M, Fangue NA, Connon RE. The transcriptome-wide effects of exposure to a pyrethroid pesticide on the critically endangered delta smelt (*Hypomesus transpacificus*). Endangered Species Research. *In press. DOI 10.3354/esr00679.*

## 8.3 Selected Oral and Poster Contributions Related to this Thesis

*Oral Presentations*

S Hasenbein, SP Lawler, J Geist, RE Connon: Pesticide mixture toxicity assessments differ between single species tests and mesocosm studies, Bay-Delta Science Conference, Sacramento, CA, October 2014

S Hasenbein, SP Lawler, J Geist, I Werner, RE Connon: Effect Assessment and Regulation of Pesticide Mixtures in Aquatic Ecosystems, California Department of Pesticide Regulation (CADPR), Surface Water Seminar, Sacramento, CA, July 2014 (invited)

S Hasenbein, SP Lawler, J Geist, RE Connon: The effects of pesticide mixtures on aquatic invertebrate communities – a mesocosm study, NorCal Society for Environmental Toxicology and Chemistry (SETAC) Annual Meeting 2014, Berkeley, CA, May 2014, 2nd place best oral presentation

S Hasenbein, SP Lawler, J Geist, I Werner, RE Connon: UCD/CDPR study: Assessing the complex effects of pesticide mixtures on aquatic communities, Pesticide Regulatory Education Program (PREP) Pesticides & Water Quality: Urban-Rural Impacts Course, Davis, CA, April 2014 (invited)

S Hasenbein, SP Lawler, RE Connon, I Werner, AK Miles, J Geist: Mesocosoms. A Tool to Assess Long-term Effects of Pesticide Mixtures on Aquatic Invertebrate Communities, Interagency Ecological Program Annual Workshop, Folsom, CA, February 2014 (invited)

S Hasenbein, SP Lawler, J Geist, I Werner, AK Miles, I Werner, RE Connon. Effects of low-level pesticide mixtures on sublethal endpoints in *Chironomus dilutus* and *Hyalella azteca*, Bay Delta Science Conference, Sacramento, October 2012

S Hasenbein, SP Lawler, J Geist, I Werner, AK Miles, RE Connon. Effect assessment of tertiary pesticide mixtures on the amphipod *Hyalella azteca* and the midge *Chironomus tentans*, Annual meeting of the SETAC Northern California Regional Chapter, Berkeley, CA, May 2012

*Poster Presentations*

S Hasenbein, SP Lawler, J Geist, RE Connon: What would fish be without food? How herbicides affect aquatic communities, Bay-Delta Science Conference, Sacramento, CA, October 2014

S Hasenbein, RE Connon, J Geist, I Werner, AK Miles, SP Lawler: A long-term effect assessment of tertiary pesticide mixtures on aquatic invertebrate communities using mesocosms, SETAC North America Meeting, Nashville, TN, November 2013

S Hasenbein, K Callinan Hoffmann, J Geist, I Werner, AK Miles, RE Connon, SJ Teh, SP Lawler: The comparative toxicity of five pesticides in *Hyalella azteca* and *Chironomus dilutus*, Interagency Ecology Program (IEP) Annual Workshop, Folsom, CA, April 2013

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# 10. Appendix

Appendix A. Results of repeated measures ANOVA conducted for chapter 4 to test the effects of treatment, time and their interaction on physicochemical parameters, the abundances of macroinvertebrates, emergence, and abundances of zooplankton. Only organisms with sufficient numbers per sampling date are listed herein.

| | d.f. | SS | MS | *F* | *P* | $\omega^2$ |
|---|---|---|---|---|---|---|
| *Physicochemical parameters* | | | | | | |
| pH | | | | | | |
| Time | 21 | 12.35 | 0.58 | 12.26 | < **0.001** | 0.36 |
| Treatment | 3 | 0.91 | 0.30 | 6.35 | **0.001** | 0.02 |
| Time x Treat. | 63 | 3.02 | 0.05 | 0.85 | 0.781 | -0.02 |
| Error | 263 | 14.88 | 0.06 | | | |
| Total | 350 | 31.22 | | | | |
| SC | | | | | | |
| Time | 20 | 10278517.00 | 513926.00 | 11.18 | < **0.001** | 0.43 |
| Treatment | 3 | 153636.00 | 51212.00 | 1.11 | 0.350 | 0.00 |
| Time x Treat. | 60 | 2757626.00 | 45960.00 | 1.28 | 0.101 | 0.03 |
| Error | 252 | 9065726 | 35975 | | | |
| Total | 335 | 22255505 | | | | |
| DO | | | | | | |
| Time | 21 | 468.55 | 22.31 | 40.07 | < **0.001** | 0.66 |
| Treatment | 3 | 2.80 | 0.93 | 1.67 | 0.181 | 0.00 |
| Time x Treat. | 63 | 35.08 | 0.56 | 0.82 | 0.824 | -0.01 |
| Error | 264 | 179.003 | 0.678 | | | |
| Total | 351 | 685.43 | | | | |
| T | | | | | | |
| Time | 21 | 6050.80 | 288.13 | 279.56 | < **0.001** | 0.98 |
| Treatment | 3 | 6.60 | 2.20 | 2.13 | 0.105 | 0.00 |
| Time x Treat. | 63 | 64.93 | 1.03 | 4.16 | < **0.001** | 0.01 |
| Error | 264 | 65.47 | 0.25 | | | |
| Total | 351 | 6187.81 | | | | |
| *Macroinvertebrates* | | | | | | |
| Total abundance | | | | | | |
| Time | 21 | 355147.00 | 16912.00 | 5.15 | < **0.001** | 0.21 |
| Treatment | 3 | 26274.00 | 8758.00 | 2.67 | 0.055 | 0.01 |
| Time x Treat. | 63 | 206769.00 | 3282.00 | 1.12 | 0.272 | 0.02 |
| Error | 264 | 775184.00 | 2936.00 | | | |
| Total | 351 | 1363375.00 | | | | |
| Gill Breathers | | | | | | |
| Time | 21 | 180304 | 8586 | 7.28 | < **0.001** | 0.26 |
| Treatment | 3 | 8114 | 2705 | 2.29 | 0.078 | 0.01 |
| Time x Treat. | 63 | 101123 | 1605 | 1.36 | 0.05 | 0.04 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Error | 264.000 | 311310 | 1179 | | | |
| Total | 351 | 600851 | | | | |
| Odonata | | | | | | |
| Time | 21 | 155301.00 | 7395.30 | 14.55 | < **0.001** | 0.48 |
| Treatment | 3 | 6613.00 | 2204.40 | 4.34 | **0.008** | 0.02 |
| Time x Treat. | 63 | 32030.00 | 508.40 | 1.21 | 0.159 | 0.02 |
| Error | 264 | 111380.00 | 421.90 | | | |
| Total | 351 | 305324.00 | | | | |
| Zygoptera | | | | | | |
| Time | 21 | 100455.00 | 4783.60 | 10.69 | < **0.001** | 0.42 |
| Treatment | 3 | 8818.00 | 2939.40 | 6.57 | **0.001** | 0.04 |
| Time x Treat. | 63 | 28203.00 | 447.70 | 1.40 | **0.037** | 0.04 |
| Error | 256 | 81904.00 | 319.90 | | | |
| Total | 343 | 221033.00 | | | | |
| Anisoptera | | | | | | |
| Time | 21 | 7324.00 | 348.76 | 4.75 | < **0.001** | 0.18 |
| Treatment | 3 | 1862.00 | 620.76 | 8.46 | < **0.001** | 0.05 |
| Time x Treat. | 63 | 4631.00 | 73.51 | 1.03 | 0.430 | 0.00 |
| Error | 244 | 17449.00 | 71.51 | | | |
| Total | 331 | 32111.00 | | | | |
| Diptera | | | | | | |
| Time | 21 | 145874.00 | 6946.40 | 6.46 | < **0.001** | 0.31 |
| Treatment | 3 | 9962.00 | 3320.50 | 3.09 | **0.033** | 0.02 |
| Time x Treat. | 63 | 67761.00 | 1075.60 | 1.47 | **0.019** | 0.05 |
| Error | 264 | 192565.00 | 729.40 | | | |
| Total | 351 | 416162.00 | | | | |
| Ephemeroptera | | | | | | |
| Time | 21 | 776.10 | 36.96 | 2.23 | **0.008** | 0.09 |
| Treatment | 3 | 305.10 | 101.71 | 6.14 | **0.001** | 0.00 |
| Time x Treat. | 63 | 1044.20 | 16.57 | 1.22 | 0.141 | -0.11 |
| Error | 264 | 3575.80 | 13.54 | | | |
| Total | 351 | 5701.20 | | | | |
| *Anopheles* sp. | | | | | | |
| Time | 21 | 18015.00 | 857.90 | 4.82 | < **0.001** | 0.22 |
| Treatment | 3 | 2408.00 | 802.50 | 4.51 | **0.004** | 0.03 |
| Time x Treat.[1] | 60 | 14992.00 | 249.90 | 1.61 | **0.008** | 0.07 |
| Error | 249 | 44324.00 | 178.00 | | | |
| Total | 273 | 64634.00 | | | | |
| *Culex* sp. | | | | | | |
| Time | 21 | 104008.00 | 4952.80 | 6.72 | < **0.001** | 0.33 |
| Treatment | 3 | 6875.00 | 2291.60 | 3.11 | **0.033** | 0.02 |
| Time x Treat. | 63 | 46449.00 | 737.30 | 1.52 | **0.013** | 0.06 |
| Error | 264 | 128050.00 | 485.00 | | | |
| Total | 351 | 285382.00 | | | | |
| Chironomidae | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Time | 21 | 2621.90 | 124.85 | 5.38 | < **0.001** | 0.19 |
| | Treatment | 3 | 167.90 | 55.96 | 2.41 | 0.075 | 0.01 |
| | Time x Treat. | 63 | 1460.70 | 23.19 | 0.91 | 0.664 | -0.01 |
| | Error | 264 | 6717.50 | 25.45 | | | |
| | Total | 351 | 10968.00 | | | | |
| Coleoptera | | | | | | | |
| | Time | 21 | 1246.86 | 59.37 | 11.38 | < **0.001** | 0.44 |
| | Treatment | 3 | 47.84 | 15.95 | 3.06 | **0.035** | 0.01 |
| | Time x Treat. | 63 | 328.66 | 5.22 | 1.35 | 0.057 | 0.03 |
| | Error | 264 | 1023.50 | 3.88 | | | |
| | Total | 351 | 2646.86 | | | | |
| *H. azteca* | | | | | | | |
| | Time | 21 | 41488.00 | 1975.60 | 5.14 | < **0.001** | 0.16 |
| | Treatment | 3 | 24415.00 | 8138.20 | 21.17 | < **0.001** | 0.11 |
| | Time x Treat. | 63 | 24222.00 | 384.50 | 1.56 | **0.015** | -0.01 |
| | Error | 154 | 64119.00 | 416.40 | | | |
| | Total | 241 | 201953.00 | | | | |
| Pulmonata | | | | | | | |
| | Time | 21 | 166589.00 | 7932.80 | 29.46 | < **0.001** | 0.33 |
| | Treatment | 3 | 4064.00 | 1354.80 | 5.03 | **0.003** | 0.00 |
| | Time x Treat. | 63 | 16963.00 | 269.30 | 0.28 | 1.000 | -0.10 |
| | Error | 264 | 257729.00 | 976.20 | | | |
| | Total | 351 | 445346.00 | | | | |
| Planorbidae | | | | | | | |
| | Time | 21 | 35851.00 | 1707.20 | 16.04 | < **0.001** | 0.14 |
| | Treatment | 3 | 1441.00 | 480.30 | 4.51 | **0.006** | 0.00 |
| | Time x Treat. | 63 | 6704.00 | 106.40 | 0.21 | 1.000 | -0.14 |
| | Error | 264 | 134554.00 | 509.70 | | | |
| | Total | 351 | 178550.00 | | | | |
| *Radix* sp. | | | | | | | |
| | Time | 21 | 58694.00 | 2795.00 | 13.41 | < **0.001** | 0.34 |
| | Treatment | 3 | 2193.00 | 731.10 | 3.51 | **0.020** | 0.01 |
| | Time x Treat. | 63 | 13129.00 | 208.40 | 0.55 | 0.997 | -0.05 |
| | Error | 283 | 95836.00 | 338.60 | | | |
| | Total | 307 | 151231.00 | | | | |
| *Emergence* | | | | | | | |
| Total abundance | | | | | | | |
| | Time | 16 | 48026.00 | 3001.60 | 22.01 | < **0.001** | 0.50 |
| | Treatment | 3 | 428.90 | 143.00 | 1.05 | 0.380 | 0.00 |
| | Time x Treat. | 48 | 6547.00 | 136.40 | 0.80 | 0.821 | -0.02 |
| | Error | 204 | 34853.00 | 170.80 | | | |
| | Total | 271 | 89854.90 | | | | |
| *Culex* sp. | | | | | | | |
| | Time | 16 | 33020.10 | 2063.80 | 16.48 | < **0.001** | 0.44 |
| | Treatment | 3 | 457.80 | 152.60 | 1.22 | 0.313 | 0.00 |

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|---|---|---|---|---|---|---|
| Time x Treat. | 48 | 6011.10 | 125.20 | 0.84 | 0.757 | -0.02 |
| Error | 204 | 30355.00 | 148.80 | | | |
| Total | 271 | 69843.90 | | | | |
| *Anopheles* sp. | | | | | | |
| Time | 16 | 497.31 | 31.08 | 2.50 | **0.007** | 0.06 |
| Treatment | 3 | 81.57 | 27.19 | 2.19 | 0.102 | 0.01 |
| Time x Treat. | 48 | 596.37 | 12.42 | 0.82 | 0.788 | -0.03 |
| Error | 204 | 3083.75 | 15.12 | | | |
| Total | 271 | 4259.00 | | | | |
| Zygoptera | | | | | | |
| Time | 16 | 805.62 | 50.35 | 6.17 | < **0.001** | 0.17 |
| Treatment | 3 | 75.22 | 25.07 | 3.07 | **0.037** | 0.01 |
| Time x Treat. | 48 | 391.97 | 8.17 | 0.69 | 0.936 | -0.05 |
| Error | 204 | 2414.75 | 11.84 | | | |
| Total | 271 | 3687.56 | | | | |
| *Zooplankton* | | | | | | |
| Total abundance | | | | | | |
| Time | 21 | 368008702.00 | 17524224.00 | 3.33 | < **0.001** | 0.12 |
| Treatment | 3 | 10630835.00 | 3543612.00 | 0.67 | 0.572 | 0.00 |
| Time x Treat. | 63 | 331765785.00 | 5266124.00 | 0.95 | 0.578 | -0.01 |
| Error | 255 | 1408183497.00 | 5522288.00 | | | |
| Total | 342 | 2102652591.00 | | | | |
| Rotifers | | | | | | |
| Time | 21 | 125507408.00 | 5976543.00 | 3.66 | < **0.001** | 0.10 |
| Treatment | 3 | 15155598.00 | 5051866.00 | 3.09 | **0.033** | 0.01 |
| Time x Treat. | 63 | 102989231.00 | 1634750.00 | 0.78 | 0.875 | -0.04 |
| Error | 264 | 550178163.00 | 2084008.00 | | | |
| Total | 351 | 793830399.00 | | | | |
| *Ascomorpha* spec | | | | | | |
| Time | 21 | 193825.00 | 9230.00 | 1.04 | 0.429 | 0.00 |
| Treatment | 3 | 37119.00 | 12373.00 | 1.40 | 0.251 | 0.00 |
| Time x Treat. | 63 | 557324.00 | 8846.00 | 1.03 | 0.431 | 0.00 |
| Error | 264 | 2275193.00 | 8618.00 | | | |
| Total | 351 | 3063461.00 | | | | |
| Brachionidae | | | | | | |
| Time | 21 | 2803165.00 | 133484.00 | 2.39 | **0.004** | 0.08 |
| Treatment | 3 | 507276.00 | 169092.00 | 3.03 | **0.036** | 0.02 |
| Time x Treat. | 63 | 3520262.00 | 55877.00 | 1.03 | 0.416 | 0.01 |
| Error | 264 | 14256314.00 | 54001.00 | | | |
| Total | 351 | 21087017.00 | | | | |
| *Brachionus* sp. | | | | | | |
| Time | 21 | 31409.00 | 1496.00 | 0.84 | 0.660 | -0.02 |
| Treatment | 3 | 10074.00 | 3358.00 | 1.89 | 0.140 | 0.01 |
| Time x Treat. | 63 | 111899.00 | 1776.00 | 0.86 | 0.757 | -0.03 |
| Error | 264 | 544145.00 | 2061.00 | | | |

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|---|---|---|---|---|---|---|
| Total | 351 | 697527.00 | | | | |
| *Keratella* sp. | | | | | | |
| Time | 21 | 220795.00 | 10514.00 | 2.12 | **0.012** | 0.08 |
| Treatment | 3 | 18996.00 | 6332.00 | 1.27 | 0.291 | 0.00 |
| Time x Treat. | 63 | 313090.00 | 4970.00 | 1.18 | 0.188 | 0.03 |
| Error | 264 | 1112397.00 | 4214.00 | | | |
| Total | 351 | 1665279.00 | | | | |
| *Trichocerca* sp. | | | | | | |
| Time | 21 | 103537561.00 | 4930360.00 | 2.99 | < **0.001** | 0.09 |
| Treatment | 3 | 16584252.00 | 5528084.00 | 3.35 | **0.024** | 0.01 |
| Time x Treat. | 63 | 103972618.00 | 1650359.00 | 0.85 | 0.769 | -0.02 |
| Error | 264 | 509777983.00 | 1930977.00 | | | |
| Total | 351 | 733872413.00 | | | | |
| *Platyias patulus* | | | | | | |
| Time | 21 | 2383659.00 | 113508.00 | 2.28 | **0.006** | 0.08 |
| Treatment | 3 | 757342.00 | 252447.00 | 5.07 | **0.003** | 0.04 |
| Time x Treat. | 63 | 3138404.00 | 49816.00 | 1.15 | 0.232 | 0.02 |
| Error | 264 | 11484628.00 | 43502.00 | | | |
| Total | 351 | 17764033.00 | | | | |
| Ostracoda | | | | | | |
| Time | 21 | 175370570.00 | 8350980.00 | 6.57 | < **0.001** | 0.19 |
| Treatment | 3 | 24342088.00 | 8114029.00 | 6.39 | **0.001** | 0.03 |
| Time x Treat. | 63 | 80047090.00 | 1270589.00 | 0.75 | 0.918 | -0.04 |
| Error | 264 | 450129907.00 | 1705038.00 | | | |
| Total | 351 | 729889655.00 | | | | |
| Cladocera | | | | | | |
| Time | 21 | 166946899.00 | 7949852.00 | 10.38 | < **0.001** | 0.34 |
| Treatment | 3 | 18264015.00 | 6088005.00 | 7.95 | < **0.001** | 0.04 |
| Time x Treat. | 63 | 48247155.00 | 765828.00 | 0.96 | 0.563 | 0.00 |
| Error | 253 | 201634225.00 | 796973.00 | | | |
| Total | 340 | 436637057.00 | | | | |
| *Bosmina* sp. | | | | | | |
| Time | 21 | 620676.00 | 29556.00 | 1.44 | 0.136 | 0.03 |
| Treatment | 3 | 23464.00 | 7821.00 | 0.38 | 0.768 | -0.01 |
| Time x Treat. | 63 | 1296831.00 | 20585.00 | 1.02 | 0.448 | 0.00 |
| Error | 264 | 5337023.00 | 20216.00 | | | |
| Total | 351 | 7277993.00 | | | | |
| Chydoridae | | | | | | |
| Time | 21 | 97303194.00 | 4633485.00 | 7.61 | < **0.001** | 0.30 |
| Treatment | 3 | 9960325.00 | 3320108.00 | 5.45 | **0.002** | 0.03 |
| Time x Treat. | 63 | 38348627.00 | 608708.00 | 1.13 | 0.247 | 0.02 |
| Error | 264 | 141662026.00 | 536599.00 | | | |
| Total | 351 | 287274173.00 | | | | |
| *Daphnia magna* | | | | | | |
| Time | 21 | 15049455.00 | 716641.00 | 3.46 | < **0.001** | 0.12 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Treatment | 3 | 9115469.00 | 3038490.00 | 14.66 | < **0.001** | 0.10 |
| Time x Treat. | 63 | 13057343.00 | 207259.00 | 1.00 | 0.490 | 0.00 |
| Error | 234 | 48632732.00 | 207832.00 | | | |
| Total | 321 | 85432456.00 | | | | |
| Copepoda | | | | | | |
| Time | 21 | 2781256.00 | 132441.00 | 2.37 | **0.004** | 0.06 |
| Treatment | 3 | 1555963.00 | 518654.00 | 9.28 | < **0.001** | 0.06 |
| Time x Treat. | 63 | 3517321.00 | 55830.00 | 0.88 | 0.722 | -0.02 |
| Error | 250 | 15854123.00 | 63416.00 | | | |
| Total | 337 | 23716338.00 | | | | |
| Cyclopidae | | | | | | |
| Time | 21 | 1895283.00 | 90252.00 | 3.08 | < **0.001** | 0.08 |
| Treatment | 3 | 1299092.00 | 433031.00 | 14.78 | < **0.001** | 0.09 |
| Time x Treat. | 63 | 1838661.00 | 29185.00 | 0.80 | 0.849 | -0.03 |
| Error | 241 | 8761148.00 | 36353.00 | | | |
| Total | 328 | 13787731.00 | | | | |
| Nauplia | | | | | | |
| Time | 21 | 290416.00 | 13829.00 | 1.91 | **0.026** | 0.04 |
| Treatment | 3 | 44413.00 | 14804.00 | 2.04 | 0.117 | 0.01 |
| Time x Treat. | 63 | 457192.00 | 7257.00 | 0.90 | 0.688 | -0.02 |
| Error | 264 | 2131258.00 | 8073.00 | | | |
| Total | 351 | 2923279.00 | | | | |

[1] = Lack-of-fit, d.f. = degrees of freedom, SS = sum squares, MS = Mean squares, F = F-ratio, P = P-value. $\omega^2$ = Magnitude of effects. Significant p-values (< 0.05) are indicated in bold.